ANIMAL HEALTH MONITORING IN THE RESEARCH ENVIRONMENT

Notes from the Workshop

This was ANZCCART's third workshop for 1998 and was co-sponsored by ANZSLAS and AVERT (Australian Veterinarians in Ethics, Research and Teaching).

The venue was the Carlton Crest Hotel, Melbourne and 46 delegates attended the workshop.

Dr Robert Baker, Director of ANZCCART, opened the workshop and welcomed the three overseas and one interstate speakers and delegates.

The workshop comprised four sessions, each with a keynote speaker, followed by general discussion.

Introduction: Dr Robert Baker.

Session One: *Animal health monitoring that is cost effective and produces meaningful results - the balance between too much and too little.* *
Dr Bill White, Charles River Laboratories, USA.

Session Two: *Development and use of transgenic rodents in preclinical research - practical issues*
Dr Patrick Hardy, Transgenic Alliance, France.

Session Three: *Importation, quarantine and monitoring of laboratory animals, particularly rodents, for issue in Australia.*
Dr Kevin Doyle, AVA, Canberra.

Session Four: *New infectious agents of rodents* *
Dr Earl Steffen, University of Missouri, USA.

* These notes were prepared from transcripts of these sessions, as written papers were not provided.

Introduction

This was the third workshop held by ANZCCART this year. It was also sponsored by ANZSLAS (Australian and New Zealand Society for Laboratory Animal Science) and AVERT (Australian Veterinarians in Ethics, Research and Teaching - a Special Interest Group of the AVA). Forty-six people attended and participated in a very interesting and worthwhile program.

There were four keynote speakers:

- Dr Bill White (Charles River Laboratories, Maine, USA)
- Dr Patrick Hardy (Charles River Pharmservices, Lyons, France)
  Dr Kevin Doyle (AVA, Canberra)
- Dr Earl Steffen (University of Missouri, USA)

The overseas speakers also made major contributions to the ANZSLAS meeting, held over the following three days. The speakers were experts in their fields and drew on their experience in presenting interesting and informative papers.

The workshop was in four sessions, each featuring one of the keynote speakers, followed by a discussion.

In session one, Dr Bill White discussed *Animal health monitoring that is cost effective and produces meaningful results - the balance between too much and too little.* His very practical paper was enlivened with delightful slides and frequent references to *White's rules of life.* He emphasised that all risks are relative (a metaphor for life) and of the need to be realistic and objective when undertaking risk assessment, which must also be cost-effective.

He addressed the importance of the questions asked by a health monitoring program and how precise the answers are required to be, what are the constraints and of the need for results to be relevant for researchers (false positive results are not helpful). Dr White stressed that when using sentinel animals, their health status must be known before their use. He ended with an excellent coverage of the use of sampling statistics.

In Session Two, Dr Patrick Hardy discussed *Development and use of transgenic rodents in preclinical research - practical issues.* He reviewed the history of transgenic animals from the first publication in 1982 to the present, where there are now over 5000 publications per year.

He discussed the Charles River facility in Lyons and how it is managed, particularly with regard to biosecurity, risk assessment and risk management. Their health monitoring program was discussed (it is very similar to that recommended by FELASA) as was the concept of SOPF animals (specific and opportunistic pathogen-free). A filter-top cage system was described, in which all conditions are as for a barrier unit, except that the staff are not required to shower. Rederivation using either embryo transfer or caesarean section was also described.

The concept of risk analysis was further developed in the third session by Dr Kevin Doyle, formerly of the Australian Quarantine and Inspection Service and now with the AVA. In his paper, I*mportation, quarantine and monitoring of laboratory animals, particularly rodents, for issue in Australia,*. he defined quarantine in Australia, from the

first Quarantine Act in 1908 through to the present. He defined risk analysis in the context of quarantine decisions about the importation of animals, as the problems of an event occurring versus the consequences if it occurs. Probability is important only with regard to the consequences.

The objective of a proposed import (e.g., of diabetic dogs for research) must be weighed against the risk (e.g., of the dogs introducing rabies to Australia).

He emphasised the need for governments to receive expert advice (from organisations such as ANZSLAS), to ensure they made the best policy decisions. Professional bodies need to lobby government and to seek representation on government advisory panels (e.g., with AQIS).

In the last session, Dr Earl Steffen very comprehensively covered the topic *New infectious agents of rodents*. He stressed the need to know whether a new infectious agent - the *agent du jour* is truly new or a known organism which has only recently been recognised. What is the significance to researchers and animal breeders and what are its morbidity and mortality rates in various species?

The majority of his paper related to specific new agents, such as the discovery of *Helicobacter* species, rat respiratory virus and scaly-skin condition in nude mice (whose aetiology is uncertain but is probably viral).

He concluded by observing that new agents are often found simply by persistent good observation, combined with serology and histology.

Session One

Animal health monitoring that is cost-effective and produces meaningful results - the balance between too much and too little.

Bill White, Charles River Laboratories, Wilmington, Massachusetts, USA.

Abstract

This presentation discusses the rationale involved in developing an institutional health monitoring program. While it is easy to emulate programs at other institutions, circumstances such as the types of investigations being conducted, physical facilities, numbers and types of species being housed, the use of bio containment or bio exclusion systems, the prevalence of specific microorganisms as well as a variety of other factors can make each institution's needs quite different. Consideration must be given to the amount and quality of information that will be massed by such a program as well as what actions will be taken, based upon the results of such a program. The concept of compartmentalisation of the health monitoring program to meet differing investigational and facility limitations will also be discussed. Sampling methodologies, as well as statistics, will be considered as well as assumptions regarding transmissibility of agents and the reliability of testing. The goal of this presentation will be to develop a sound foundation and recommendations for health monitoring which can be tailored to institutional needs in a cost effective manner.

Definitions

Dr White opened his talk with a number of definitions.

- **Axenic (or germ-free)** animals. These are supposed to be devoid of all other life-forms. They are not commonly used, are difficult to maintain and their health monitoring is very intensive.

- **Specific pathogen free (SPF)** animals. These are free of at least one organism. An SPF animal can be free of any number of specified organisms. The amount of health monitoring is therefore dependent on what organisms need to be excluded.

- **Conventional** animals. These are groups of animals whose health status is not known.

- **Epizootic**. This refers to when an organism gains entrance into a naive group of animals and can then undergo rapid dispersion in the population, which is often manifested by clinical effects. Once it has become established, the clinical effects are much less common.

- **Zoonotic**. This indicates that there is a possibility of transfer of an organism from animals to man or from man to animals.

- **Morbidity**. This is the percentage of the total population affected by an organism.

- **Mortality.** This refers to the percentage of affected animals which die from that infection.

An infectious organism is a "wild card", an experimental variable which can cause overt disease or mortality, alter research findings, contaminate biological materials and which may present a health hazard for laboratory staff - i.e., by being potentially zoonotic.

While this provides the rationale for health monitoring of laboratory animals and in particular, of laboratory rodents, there are in fact many organisms which people wish to exclude from their animals but which they are incapable of excluding. A recent survey in the USA showed a high percentage infection of one or more common organisms in non-SPF mice in one or more rooms of many animal facilities.

Dr White advised giving priority to removing basic problem organisms (e.g., pin worms, mites or MHV) before worrying about very unusual or opportunistic organisms (Jacoby and Lindsey, 1997).

The same study also surveyed SPF-mice and found that pathogenic helicobacters were present in more than 10% of colonies. Other organisms present included mycoplasmas, parvovirus and a variety of parasites. But while parasites are undesirable, interactions are few. A balance therefore has to be found between eliminating organisms whose presence is unlikely to affect research results against those which may have profound effects on research data.

Hazards and risk assessment

The job of any health care professional in laboratory animal medicine is to understand the risks imposed by organisms and to come to some consensus with their institution or department as to exactly what importance should be placed on various organisms. It is necessary to define the risks and to realise that all risks are relative.

A health monitoring program must focus on the particular institution's needs. What may be a risk for one institution may not be for another. Many rodent organisms do not often cause clinical disease, but often cause sub-clinical infections with no histological changes. If clinical signs are observed they are usually in a naive population or in an epizootic phase of infection. Sub-clinical infections generally only have limited or subtle research effects. Such infections are usually self-limiting, after protective antibodies have been produced.

Dr White emphasised the need for caution in interpreting case reports, as commonly screened - for organisms have often been selected for on an historical basis, rather than through an analysis of risk and prevalence. His message was that, while a lot of people can supply recommendations, in the end each institution has to develop its own philosophy regarding monitoring for disease in its laboratory animals. A realistic assessment of research programs, animal care programs, personnel and facilities, is required by each research establishment. This includes assessment of the existing barriers and of their effectiveness in controlling movement of organisms from room to room. It also includes the development of an institutional exclusion list, consisting of microorganisms of particular concern (e.g., mycoplasma). In specific projects or project areas this list may be extended. Such an approach allows the health monitoring program to be focused on those areas which require the greatest amount of monitoring, rather than trying to apply the standard across the whole institution and possibly wasting a lot of resources. He advised against adopting the most

stringent requirements for the whole institution, but recommended the development of questions which are essential for the health monitoring program to answer. The more specific the questions, and the more detailed the information required, the more expensive it will be.

The cost/benefit of testing is the key point, as available funding is always limited. The biggest risk, once a stable population of laboratory animals has been established, is the addition of new animals.

A disaster plan needs to be developed for each organism on the exclusion list. Once an unwanted organism is identified, there needs to be an action plan detailing what to do next. This should include consultation with the investigator utilising the animals, with whom agreement should be reached regarding the course of action to be followed.

Dr White stressed the need "to be smart about selecting testing methods, sampling frequencies and numbers and what will be sampled .....You can be too general, too complete." It is necessary to assign ownership of the health monitoring program. This should include decisions regarding:

- who is going to pay for the program?
- who is going to take the samples?
- which animals/colonies/room are to be tested?
- who ensures that the samples are sent to the laboratory?
- who looks at the results?
- who keeps the records?
- who notifies whom?
- who makes the decisions?

Investigators need to understand why particular organisms need to be excluded. Once again, the question of the cost of testing versus benefits to be gained from their identification and subsequent exclusion must be discussed with investigators and consensus reached before a testing program is begun.

It is generally true to assume that the more specific the question, the more expensive the answer. Conversely, the more general the question, the less costly is the answer.

Another important factor to consider is the physical limitations imposed by the facility. To truly separate groups of animals there must be an intact physical barrier between them. There cannot be shared communication, shared air, or shared equipment between different groups of animals. All such equipment should be decontaminated or disinfected. But it is difficult so assure sterilisation, particularly with reference to viruses.

Sharing research equipment between laboratories without adequate disinfection is another sure way of spreading unwanted organisms. This needs to be understood by all persons, especially newcomers such as graduate students.

The type of animal housing to be used should be based on the extent of biosecurity required; including whether the animals are immuno-competent or immuno-deficient.

While cost is a major concern when deciding which organisms are to be excluded, it should not be a factor when ensuring the security of animals in a micro-isolation cage or in some other form of isolation. Cost has to be balanced against the degree of

confidence required that the colony is free of specified organisms. For example, a 95% confidence limit in this context means that in one in 20 times, the animals will be contaminated with the unwanted organism, with the possibility that results may be affected. In other words, the cost must be balanced against the value of the animals as well as the cost of the experimental work performed on them.

The phrase "period of vulnerability" was emphasised by Dr White. This refers to the time from when the animals were last found to be negative to when they are diagnosed as positive. This also has cost implications for the animal house, as animals supplied during this period may not be able to be used and may have to be replaced, with costs being borne by the supplier. The longer an organism remains undetected in an animal population, the more widespread it becomes and the more difficult and expensive it will be to eradicate. This will therefore influence the frequency of testing for unwanted organisms.

Infectious agent monitoring plan

Dr White referred to his tables (tables 1-6) which list a variety of organisms (viruses, bacteria and parasites) for mice, rats, guinea pigs, rabbits, gerbils and hamsters, compared with a number of possible actions, depending on the immune status of the animal, amd whether housed in micro-isolators or in an animal room.

Table 7 provides definitions of actions, based on six categories of risk (A to F). These provide a guide to animal house staff for assessing risk and determining courses of action for different pathogens in the common laboratory animal species. He emphasised that not all organisms are important and that an institution needs to have an order of importance for organisms found in its animals.

He discussed selection criteria for pathogenic organisms. Questions to be asked include:

- is the agent a primary rodent pathogen?

- is it capable of epizootically infecting rodents and producing direct or indirect evidence of its presence in a significant proportion of the population?

- does an enzootic infection result in a demonstrable clinical or histological disease in a significant proportion of the population and are rodents a primary preferred host? It is important to know whether humans also carry the organisms - e.g., *Staphylococcus aureus*, which is commonly associated with acne. *Pseudomonas aeruginosa* is a common contaminant of domestic water supplies and is a common human commensal organism.

- has the pathogenic potential of the organism in rodents been established before commencing an expensive bio-exclusion program? It is difficult to evaluate the pathogenic potential of many organisms, especially opportunistic bacteria.

This is relevant when considering case reports, comprising identification or serotyping of organisms from a sample of animals. Detailed identification of organisms is often not done well if at all and procedures to exclude other agents are often not carried out. A complete histo-pathological examination is often absent from case reports, as is a complete evaluation of contributing host factors, and Koch's postulates are seldom satisfied.

- does the organism have an established effect on research when present?

- other than through clinical signs or death, can the organism interact with metabolic, immunological or physiological processes in a way which has been adequately described and published in the literature? If this is not the case, it is very difficult to explain to investigators how this organism may actually affect research results.

- is the agent either ubiquitous in the environment or commonly associated with normal human flora? If so, it is going to be very difficult to keep out.

- if there is an organism which must be excluded, what is the likelihood of achieving this through rederivation techniques as well as barrier production methods? Can it be kept out once it has been excluded?

The availability and reliability of detection methods is very important. Can an organism be reliably detected if present? There are some primary pathogens which have no research interactions. There are occasional pathogens with suspected research interactions, opportunistic pathogens with direct research interference only under special conditions and then there are the bacteria whose function and pathogenicity (if any) are not known.

Developing a health monitoring program

There are two approaches:

- diagnostic health monitoring, which is retrospective.

- routine health monitoring, which is prospective.

The former is only an imperfect early warning system, which can provide false positive and/or false negative results. It is very unsatisfactory to cull a lot of animals on the basis of a set of false positive results. Unusual findings should be coupled with regular clinical observation and mortality records.

The goal of any screening program is to detect the presence of an organism in a group of animals by detecting its presence in one animal. The goal is **not** to determine the morbidity or prevalence of the infection in the population. You only have to find one positive for the population to be positive. Sampling is about statistics - only one positive means the incidence in the population is low. To know the status of a colony, you need to sample enough of the appropriate animals on a significantly frequent basis. Animals selected should be representative of the colony as a whole. Truly random selection (if possible) is best and should take into account differences in age, strain, sex and susceptibility. Sampling therefore should be rotated among various strains, ages and sexes, as well as being from different locations in the room. As many agents are particulate-borne, gravity and electrostatic charge need to be considered. Mites tend to feed on dust and bedding particles on the lower shelves of racks and so animals from these cages can serve as sentinels for external parasites.

For detection of organisms, including viruses, immunologically competent animals of at least eight weeks of age are used. If using sentinels, their health status must be known before introducing them to the program. They will need at least four weeks' exposure to a colony and there has to be some form of contact between animals to

allow an organism to spread. This can be by direct animal contact (usually not possible) or via soiled bedding.

Dr White discussed open cages versus filter-top cages and noted that shedding organisms are a risk in open cages. Other species can be used as sentinels, e.g. guinea pigs.

For pathology, bacteriology and parasitology animals of various ages are used, as the prevalence of infection with some bacteria or parasites is age-dependent. Enteric protozoa occur more commonly in weanlings rather than in older rodents. Charles River Laboratories, USA usually screens 16 animals per room using multiple age groups. Immuno-suppression may also be used to confirm diagnoses. There is no simple answer to determining sampling frequency, which is a balance between resources and the acceptable period of vulnerability. The frequency will vary according to the type of housing, so the more defined the animals and the more complex the housing, the more frequent is the need for sampling, to be assured that the health status of the colony is being maintained.

A single sample is only a snapshot at one point in time of the colony. The minute after the samples are taken, an unwanted organism can enter, or if already present, can become detectable. But each snapshot is expensive.

Sampling schedules for screening of different organisms can be different and are dependent on the prevalence of the organism in the research animal community, in that region or country, the institution's view on the organism's role or importance and on whether certain populations are at greater risk than others.

A rapid turn-over of animals in a facility may actually decrease the risk of an organism becoming established in a colony. If animals only spend three to six weeks in the facility, the risk of spread to the population may be smaller and hence the health monitoring program may not need to be as intensive.

Once an organism enters a facility, it takes a while for it to move through, based on how the organism multiplies within animals and how rapidly it is shed.

Statistics and sampling techniques

It is necessary to determine the limits and assumptions on a population to be sampled to determine the sample size. Most sampling statistics are based on the assumption that the unrestricted population is 100 or more. The use of bio-exclusion by a containment device in many cases will restrict the population to less than 100, causing each cage to effectively become a separate population.

In the case of populations greater than 100 to over 300,000, the sample size required does not increase in proportion to the size of the population, but remains the same as if it was 100. The goal is to detect the presence of an organism in the population by finding one positive animal, not determining what is the actual morbidity. However, while it is generally best to use a random distribution, this is not always true. There can be variations in susceptibility to various organisms between sexes and strains, which may affect how quickly animals acquire an infection. While it is assumed that the sampling method will detect the agent and that the testing method will be 100% specific and 100% sensitive, this of course is not always the case and can lead to false positive or false negative results.

The most common calculation is the binomial calculation of sample size, but this does not apply in populations of less than 100. A correction factor needs to be applied to small populations, as the sample size is significantly larger than predicted by the binomial. In other words, fewer animals will require a larger sample size.

Sampling formulae do not assess how often one should test. If the results are negative now, how confident can you be that in one week or one month they will still be negative? This can be ascertained by confidence projection over time, using historical records of contamination coupled with sampling frequencies and population numbers. This is called a cumulative hazard frequency. These rates are institutionally dependent and can be calculated for any institution which has had a health monitoring program in place for some time and which has kept good records. Most institutions in the USA look at five to ten samples from large open colonies, with screening done infrequently, usually quarterly.

When sample size is at or below the size needed to detect the organism reliably at a given morbidity, you should:

- increase sample size;
- increase sample frequency; and
- wait until morbidity increases, assuming the agent is capable of achieving a higher morbidity.

Although pooling of samples may save money on laboratory testing, it does not make sense with bacteriology. If there is an overgrowth of bacteria, you cannot tell what has been missed. Some bacteria will suppress growth of other bacteria, which may also be missed during culture.

Screening frequency was discussed above, with the observation that most laboratories screen quarterly. Some agents can be screened for less frequently, perhaps only once or twice a year. Frequency of screening and the list of infectious agents under scrutiny may be different for different colonies and for different areas in the same institution.

If an organism cannot be excluded, why screen for it? Do not screen for any organism unless there is a plan developed to eradicate it. Don't screen for anything you are not prepared to eliminate from your facility. Don't screen for agents for which there is no sound and reviewed justification.

Use the routine screening program to answer the basic questions and the diagnostic screening program to acquire detailed information needed to characterise the situation. Do not act on any results which have not been confirmed or which do not make sense. Beware of false positive results. These can be discounted by repeated findings of one or more positive animals over time, or of an increasing number of positive samples over time.

Always be practical and remain conscious of cost.

Reference

Jacoby, R.O. and Lindsey, J.R. (1997) Health care for research animals is essential and affordable. *Health Care for Research Animals* **11:** 609-614.

Table 1 Infectious Agent Monitoring Plan

Species: Mouse

		Action Code			
		Immunocompetent		Immunodeficient	
Category	**Agent**	**Micro-Isolator**	**Animal Room**	**Micro-Isolator**	**Animal Room**
Viruses	Sendai				
	PVM				
	MHV				
	MVM				
	GD-VII				
	REO 3				
	EDIM				
	LCMV				
	Polyoma				
	MCMV				
	Ectromelia				
	MPV(OPV)				
	MAD				
	K				
	MTLV				
	Hantavirus				
Bacteria	CAR bacillus				
	B. bronchiseptica				
	C. freundii 4280				
	C. kutscheri				
	Salmonella spp.				
	M. pulmonis				
	S. moniliformis				
	H. hepaticus				
	K. pneumoniae				
	K. oxytoca				
	P. multocida				
	P. pneumotropica				
	Pasteurella spp.				
	P. aeruginosa				
	Pseudomonas spp.				
	Staph. aureus (coag+)				
	Strep. pneumoniae				
	ß. Strep. spp. Group B				
	ß . Strep. spp. Group G				
	ß . Strep. spp.				
Parasites	Ectoparasites				
	GI-Helminths				
	GI-Protozoa/sporozoans				
	E. cuniculi				
Pathology	Gross Examination				

Table 2 Infectious Agent Monitoring Plan

Species: Rat

		Action Code			
		Immunocompetent		Immunodeficient	
Category	**Agent**	**Micro-Isolator**	**Animal Room**	**Micro-Isolator**	**Animal Room**
Viruses	Sendai				
	PVM				
	SDA/RCV				
	KRV				
	HI				
	REO 3				
	LCMV				
	HANT				
	MAD				
	RPV (OPV)				
Bacteria	CAR bacillus				
	B. bronchiseptica				
	C. kutscheri				
	Salmonella spp.				
	M. pulmonis				
	S. moniliformis				
	H. hepaticus				
	K. pneumoniae				
	K. oxytoca				
	P. multocida				
	P. pneumotropica				
	Pasteurella spp.				
	P. aeruginosa				
	Pseudomonas spp.				
	Staph. aureus (coag+)				
	Strep. pneumoniae				
	ß. Strep. spp. Group B				
	ß . Strep. spp. Group G				
	ß . Strep. spp.				
Parasites	Ectoparasites				
	GI-Helminths				
	GI-Protozoa/sporozoans				
	E. cuniculi				
Pathology	Gross Examination				

Table 3 Infectious Agent Monitoring Plan

Species: Guinea Pig

		Action Code			
		Immunocompetent		Immunodeficient	
Category	**Agent**	**Micro-Isolator**	**Animal Room**	**Micro-Isolator**	**Animal Room**
Viruses	Sendai				
	PVM				
	REO-3				
	LCMV				
Bacteria	*B. bronchiseptica*				
	Salmonella spp.				
	M. pulmonis				
	S. moniliformis				
	S. zooepidemicus				
	K. pneumoniae				
	K. oxytoca				
	P. multocida				
	P. pneumotropica				
	Pasteurella spp.				
	P. aeruginosa				
	Pseudomonas spp.				
	Steph. aureus (coag+)				
	Strep. pneumoniae				
	ß. Strep. spp. Group B				
	ß . Strep. spp. Group G				
	ß . Strep. spp.				
Parasites	Ectoparasites				
	GI-Helminths				
	GI-Protozoa/sporozoans				
	E. cuniculi				
Pathology	Gross Examination				

Table 4 Infectious Agent Monitoring Plan

Species: Rabbit

		Action Code			
		Immunocompetent		Immunodeficient	
Category	**Agent**	**Micro-Isolator**	**Animal Room**	**Micro-Isolator**	**Animal Room**
Viruses	Rotavirus				
	PI-1				
	PI-2				
	REO-3				
Bacteria	CAR bacillus				
	B. bronchiseptica				
	Salmonella spp.				
	P. multocida				
	Pasteurella spp.				
	P. aeruginosa				
	Pseudomonas spp.				
	C. perfringens				
	C. piliforme				
	Treponema cuniculi				
Parasites	Ectoparasites				
	Hepatic coccidia				
	Metazoa				
	Intestinal coccidia				
	Other protozoa				
	E. cuniculi				
Pathology	Gross Examination				

Table 5 Infectious Agent Monitoring Plan

Species: Gerbil

		Action Code			
		Immunocompetent		Immunodeficient	
Category	**Agent**	**Micro-Isolator**	**Animal Room**	**Micro-Isolator**	**Animal Room**
Viruses	LCM				
Bacteria	*B. bronchiseptica*				
	Salmonella spp				
	M. pulmonis				
	K. pneumoniae				
	K. oxytoca				
	P. multocida				
	P. pneumotropica				
	Pasteurella spp.				
	P. aeruginosa				
	Pseudomonas spp.				
	Staph. aureus (coag+)				
	Strep. pneumoniae				
	ß. Strep. spp. Group B				
	ß . Strep. spp. Group G				
	ß . Strep. spp.				
	C. piliforme				
Parasites	Ectoparasites				
	GI-Helminths				
	GI-Protozoa/sporozoans				
Pathology	Gross Examination				

Table 6 Infectious Agent Monitoring Plan

Species: Hamster

		Action Code			
		Immunocompetent		Immunodeficient	
Category	**Agent**	**Micro-Isolator**	**Animal Room**	**Micro-Isolator**	**Animal Room**
Viruses	Sendai				
	PVM				
	REO-3				
	LCM				
Bacteria	*B. bronchiseptica*				
	Salmonella spp.				
	M. pulmonis				
	Campylobacter jejuni				
	Campylobacter spp.				
	K. pneumoniae				
	K. oxytoca				
	P. multocida				
	P. pneumotropica				
	Pasteurella spp.				
	P. aeruginosa				
	Pseudomonas spp.				
	Staph. aureus (coag+)				
	Strep. pneumoniae				
	ß. Strep. spp. Group B				
	ß . Strep. spp. Group G				
	ß . Strep. spp.				
Parasites	Ectoparasites				
	Demodex				
	E. cuniculi				
	GI-Protozoa/sporozoans				
	GI-Helminths				
Pathology	Gross Examination				

Table 7 Infectious Agent Monitoring Plan

I Action Code Definitions

Category	Inform Investigators	Stop Future Receipt	Isolate Infected Animals	Test All Groups	Depopulate and Disinfect	Isolate Non-Infected Animals
A	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**No**
B	**Yes**	**Yes**	**Yes**	**No**	**Yes**	**No**
C	**Yes**	**Yes**	**Yes**	**No**	**No**	**No**
D	**Yes**	**No**	**Yes**	**No**	**No**	**No**
E	**No**	**No**	**No**	**No**	**No**	*
F	‡	‡	‡	‡	‡	‡

* Only if critical to research project.

‡ Insufficient information to make an informed judgement

II Risk Category

Category A Presents a well-documented risk to most research or is a human zoonotic risk

Category B Presents a well-documented risk to certain types of research or research projects.

Category C Is an opportunistic organism or an environmental commensal with only occasional or minimal effects on some research projects; its effects are poorly studied.

Category D Is an opportunistic organism that rarely has any research effects; and when it does, it only causes them in a few isolated animals or groups of animals.

Category E An organism that poses no documented research risks or whose effects are poorly substantiated (single case report).

Category F An organism that has not been well-studied in this species or under these circumstances.

Session Two

Development and use of transgenic rodents in preclinical research - practical issues

Patrick Hardy, Transgenic Alliance, Charles River - Iffa Credo, Les Oncins, B.P. 0109, F-69592 L'Arbresle Cedex, France

Abstract

The development of transgenic technology and the sky-rocketing increase in the generation of transgenic and target mutant rodents have serious consequences on the global management of laboratory animal resources: frequent and multiple new projects, need for additional housing capacity and containment systems and increased health monitoring and genetic testing.

More than ever, it is critical to provide the investigator with high quality animal models and an adequate level of service.

One of the main challenges is to develop an adequate, efficient and cost-effective global health management and monitoring system, in accordance with the European regulations governing the use of animals for scientific purposes and the contained use of genetically modified organisms.

The integrated containment system developed at Charles River Europe - Transgenic Alliance Department over the past five years has been successfully used for the health management of more than 200 different projects during that time.

Introduction

Transgenic Alliance is a dedicated European Department of Charles River Transgenic Services, USA. It is mainly service oriented and employs about 95 staff. Its role is to develop, breed and maintain transgenic lines under confidential agreements with universities and pharmaceutical companies. This is a very fast-growing activity, with three or four new transgenic lines received each week.

There are several fields of application of transgenic technology. If the focus is on biomedical research, (and most papers published relate to this field), there are two different and opposite applications. These comprise a multitude of small projects covering many different areas of scientific interest and a few very large projects, which can be very complex to manage.

Practical issues

Use of transgenic models in drug discovery

Laboratory animals have been intensively used in drug discovery and biomedical research. Their contribution to progress in understanding and curing diseases is undoubtedly a major one. The continuous need to improve the quality, consistency and reliability of experiments involving animals, combined with related animal welfare aspects, has generated the discipline of laboratory animal science and

technology. This addresses topics such as health quality and monitoring (microbiology, parasitology, virology, infectious pathology, serology, hygienic prophylaxia), genetic standardisation and monitoring (inbred and outbred stocks) genetics, genetic monitoring, security and reference colonies management, environment control (nutrition and diet technology, control of housing conditions, caging and enrichment), experimental techniques, animal care and handling, anaesthesia and euthanasia.

Some historical milestones in the evolution of laboratory animal science and technology are :

- gnotoxeny and isolator technology;
- creation and use of the Specific Pathogen Free concept in breeding with the related health monitoring schemes;
- increasing use of inbred strains due to the development of immunology;
- progress in rodent pathology, and
- genetic management of outbred colonies.

The most recent significant evolution (some even call it “revolution” or “break-through”) is the availability of transgenic animals (an expression of a transgenic construct micro-injected into an oocyte pronucleus), of target-mutants (from gene alteration or inactivation following homologous recombination) and of conditional mutants (Cre-Lox constructs) made possible by the advances in molecular biology. In this paper, the word transgenic includes all these categories of genetically modified animals.

These novel systems are still considered laboratory animals. The historical lessons and experience accumulated with the classical animal models (outbred and inbred genetics, isolator technology, gnotoxeny, health and genetic monitoring, large scale SPF breeding), other mutants (spontaneous, induced) and pathological models are still highly relevant !

Animal welfare and ethical aspects

While animal welfare, ethics, public perception and communication issues are not covered in this presentation, they are probably amongst the key factors which will influence the future use of transgenic technology and models.

Custom development versus standard catalogue animal models

If patents, industrial property rights, access fees and royalties do not prevent any further development, a new animal line identified as a potential pharmacological or toxicological model should present a significant improvement over existing models, have an expected life cycle of at least three years and address a reasonably large field of use. However, these conditions often cannot be fulfilled and a commercial breeding company will not be in a position to finance the model's development and support its validation and characterisation.

The most frequent alternative to use of a standard animal model is a custom designed model with creation, development and animal breeding under exclusive and confidential agreement and totally supported by one or several sponsors. In Europe, multinational research networks are now involved in co-development and use of research models. A future development will be involvement in the full project,

starting with the design and creation, followed by development, validation, characterisation, colony security and large scale breeding.

The current trend seems to favour the outsourcing of most transgenic services - pharmaceutical companies and even some governmental institutions often prefer to focus on their main mission and to concentrate all their strength on "pure" research activity. Several reasons support this situation:

- full dedication of personnel and technical resources to research projects;
- reduction of capital expenditures;
- priorities management;
- need for flexibility (the programs are subject to frequent revisions) and short set-up time;
- cheaper cost of outsourced projects;
- inadequate or unavailable animal resources (size or quality);
- management of sanitary risk (biosecurity concerns linked to the multiple and frequent infectious contaminations of transgenic lines);
- administrative burden due to regulations (use of animals in scientific procedures, genetically modified organisms); and
- company image.

Availability of existing models

When a decision is made to work with an already existing transgenic line, the project can be delayed for reasons such as:

- a patent protection, licensing agreement or financial conditions prior to access to the use of a line (e.g., access fee before any available validation evidence, royalties on any new drug developed using the animal model);

- problems such as poor reproduction, early mortality impairing breeding, lethal homozygocity for knock-out lines, unavailability of founders' animals because of the demand; and

- physical availability of the line. There is often a waiting list of several weeks or months with no guarantee about the shipment date and actual availability. There can also be major difficulties in organising the transfer of the animals.

International repositories are playing a key role in advising owners of transgenic lines, in managing the availability and distribution of potential models and preventing the duplication of line generation.

Fields of use and quality requirements

Transgenic technology has generated several industrial applications :
- plant and livestock improvement;
- use of "gene farming" for therapeutic protein production;
- vaccine engineering;
- xenografts; and
- development of gene therapy protocols.

This paper focuses on the use of transgenic rodents in biomedical research. Such uses include :

- models in pharmacology and toxicology ;
- models of human genetic diseases (e.g., cystic fibrosis, myopathies);
- models of complex human diseases, characterised by multiple aetiologic factors (e.g., Alzheimer's disease) and atherosclerosis.

Most of the current applications are in fundamental research in genetics, developmental biology, immunology, oncology, neurosciences and virology.

A smaller number of models are used in preclinical drug discovery. Much larger numbers of animals are required to meet the statistical requirements. The potential contribution of transgenic technology and emerging models to the rational drug discovery approach is very important for the pharmaceutical industry.

For models in pharmaceutical research and development, specific issues need to be addressed. These include:

- the need to rely on more relevant models (requiring creation of new animal models or improvement of the already existing ones) which mimic human diseases and the effects of the reference drugs;
- the need to develop innovative toxicology models able to increase the predictability of toxic effects in humans, to allow earlier detection of lesions and a subsequent reduction of study duration and costs;
- their availability and consistency over a long period of time;
- absence of risk to transmit any known infectious disease to man or other laboratory animal species;
- absence of any identified infectious agent likely to interfere with breeding and research;
- optimal life conditions and life span even with highly sensitive animals, and long term availability of high value models;
- a fully standardised genetic background, allowing a reliable and consistent expression of the transgene or the mutation (the phenotype can be greatly affected and modified by the genetic background);
- the isogenicity of individuals within the same line, a minimum genetic background drift from one generation to the other and the availability of a congenic control line. (This crucial step is too often neglected or not considered);
- model characterisation and validation with currently used investigation techniques and reference drugs;
- the quantity of animals available (number of groups and number of individuals per group) every week or every month, with a defined sex, age or weight bracket, to meet the statistical requirements;
- breeding performances, with a life span and reproductive life compatible with the expected breeding level and timing;
- a breeding system to optimise cost, productivity and quality;
- other factors such as housing and caging systems, genetic status (homozygous or heterozygous), mating scheme and diet;
- the possible requirement to sign a licence agreement for access to the line, under reasonable and acceptable conditions (including financial, considering the global estimated value of the models);
- access to the model waiting lists, shipment conditions (considering animal welfare and genetically modified organisms containment requirements), and
- cost of shipment.

Operations related to health standards

The following operations are usually carried out :

- reception and quarantine in negative pressure isolator (biosecurity management);
- assessment of health quality and sanitary risk ;
- re-derivation by aseptic hysterectomy or embryo transfer ;
- transfer of standard flora (Specific Pathogen Free (SPF) or Specific and Opportunistic Pathogen Free (SPOF));
- maintenance of a health security colony (live and / or cryopreserved) ;
- breeding under adequate microbiological conditions; and
- regular health monitoring according to the defined health status.

Operations related to genetic standards

Examples of operations related to the transgene or to the genetic background include:

- backcross to an inbred background (reduction of genetic variability in experimental groups and through generations, availability of a control line);
- breeding to homozygocity ;
- production of a transgenic F1 hybrid;
- production of multiple pathological models (combining transgenic constructs, target or spontaneous mutation, experimental inductions);
- genetic testing (see below);
- phenotypic testing (protein, enzyme, receptor, hormone, neuromediator detection or dosage) :
  - as a validation of the genotyping procedure ;
  - as the selected routine testing ;
  - as a confirmation assay, in addition to genetic testing (e.g., for future breeders); and
- maintenance of a genetic security colony (live and / or cryopreserved).

Operations related to security colonies

Independently, or in addition to other operations, the following should be carried out:

- maintenance and monitoring of a living colony in an isolator or in a filter-top cage system ;
- collection, freezing and storage of cryopreserved embryos or sperm; and
- thawing and reimplantation of embryos or in vitro fertilisation.

Health monitoring and genetic testing

Health monitoring schemes should be employed at these times and for the following reasons:

- before and after re-derivation ;
- specific pathogens and opportunistic microorganisms (standard or extended FELASA list, reduced screening);
- necropsy, parasitology, bacteriology, serology, qualitative or quantitative PCR techniques ;
- screening frequency (weekly, monthly or quarterly) ; and
- sample size.

Genetic testing

- protocol development or transfer validation, future breeder selection, routine screening of experimental animals;
- Southern blots, PCR, Slot Blot, qualitative or quantitative DNA amplification techniques (standard or Taq Man PCR), RFLP / DNA profile;
- transgene or mutation genotyping, zygocity testing, background characterisation or comparison; and
- speed backcrossing using DNA profile with standard proprietary probes and selected restriction enzymes.

In addition to the testing of models developed and bred by this company's transgenic services department, these health monitoring and DNA testing services are also used for animals developed, maintained or bred in experimental facilities. They can dramatically enhance the investigator's ability to monitor and characterise their laboratory animals.

Genetic testing techniques

The following techniques (based on sequence or length DNA polymorphism) are currently used when managing a project or upon receipt of biopsies for DNA testing :

- DNA profile testing (also called DNA fingerprinting or RFLP assay) , with the Charles River Therion proprietary multi-locus or single locus probes, for monitoring of strain identity (genetic background) and genetic drift. A DNA profile assay of 40-60 genetic markers will take about three to five weeks. This technique is now replacing the conventional protein assays and skin graft tests.

- Microsatellite testing is used for routine authentification of inbred and F1 hybrid genetic backgrounds. A set of 16 murine microsatellite PCR primers has been selected (eight for mice and eight for rats), which survey eight independent loci. Species- or strain-specific microsatellite primers may also be developed.

- Transgene or mutation identification is probably the most frequent assay, to test for gene carrier status and zygocity in transgenic and knockout lines, utilising DNA amplification like standard or Taq Man PCR techniques, Southern and slot blots.

- Protocol development or validation may be necessary when transferring or optimising an existing assay, or in order to develop a new protocol for transgenic or knockout lines.

These services are also available for non-rodent species such as primates, rabbits, guinea-pigs and dogs.

Other operations

- management of import, licensing, collaboration agreements;
- participation to the experimental validation process and to the model characterisation;
- improvement of breeding process (productivity, cost);
- export, shipment organisation;

- set-up of a production colony closer to the investigator's premises (e.g., in sister companies); and
- individual identification, special diet or treatment.

Health management and monitoring, bioexclusion and biocontainment

The development of transgenic technology and the rapid increase in the generation of transgenic and target mutant rodents have serious consequences for the global management of laboratory animal resources. These include frequent and multiple new projects, the need for additional housing capacity and containment systems, increased health monitoring and genetic testing.

More than ever, it is critical to provide the investigator with high quality animal models and an adequate level of service.

One of the main challenges is to develop an adequate, efficient and cost-effective global health management and monitoring system, compatible with the European and other regulations governing the use of animals for scientific purposes and the contained use of genetically modified organisms.

The integrated containment system developed at Charles River Europe – Transgenic Alliance Department over the past five years has been successfully used for the health management of more than 200 different projects during that time.

The following notes illustrate this global containment and health management system, then focus on the comparison between the use of isolators and a filter top cage system.

The challenge

When managing multiple transgenic projects as a commercial service, it is vitally important to combine the long-term goal of health management throughout the facility with the short-term flexibility and capacity to introduce new and often contaminated founder animals. The latter may originate worldwide, with no or poorly reliable health monitoring (and genetic testing reports). Management is essential if new projects are to be initiated on a regular basis without endangering the health status and breed performance of previously re-derived and well-established colonies.

The management of a large-scale transgenic rodent facility is a complex undertaking, combining several critical and sometimes conflicting factors , such as:

- the need for high capacity and flexibility (variable caging capacity, small and large scale projects, a quick set-up time and reactivity);
- a large number of projects and sub-projects with frequent modifications to protocols;
- the control of labour-intensive and complex operations (identification, biopsies, injections, frequent observation and handling;
- the variety of different models and of genotyping procedures ;
- the reduction of running costs ; and
- the need for long-term genetic and health quality of high added-value animal models carrying a high quality flora.

Biosecurity management / biocontainment upon reception and for short term / small scale projects

On reception, considering the risk of contamination by various pathogens, the frequency of shipments and project set-ups, the poor reliability of health reports, unscheduled or late deliveries, the variable shipping conditions and crate quality, this company relies exclusively on the use of negative pressure isolators until a reliable health report is available.

For most projects, it is compulsory to carry out a re-derivation (generally by embryo transfer or sometimes with outbred stocks to obtain germ-free animals by aseptic hysterectomy) and transfer of a suitable standard flora. We are currently using our SOPF flora, which has been selected to exclude the major opportunistic bacterias, to benefit from a efficient barrier flora and to facilitate the health monitoring program.

After re-derivation, the colony is maintained in a positive pressure isolator until reception of the health report and validation of the rederivation. At this stage our alternative containment system can be considered.

For short term or small scale projects the original health status (when "SPF like") may be accepted for experimental use. In such a situation the isolator can simply be switched from negative to positive pressure.

An alternative to the isolator for bioexclusion : the Filter Top Cage (FTC) system (see Table 1)

Isolator containment is the most reliable method. However, when managing a long term and / or large scale project, one may use a containment system more compatible with a high number of animals, frequent experimental procedures (labour intensive projects) and budget issues (both capital expenditures and running costs).

This company has validated and routinely used an FTC system since 1983 without any contamination to breed about 500 000 mice. Initially it was used for the large scale and low cost production of SOPF nude mice with the absence of any opportunistic infections observed in classical barrier units (e.g., conjunctivitis, subcutaneous abscesses, septicaemia and wasting syndrome).

When it was necessary to select a housing and containment system for large scale / long term transgenic projects, it was decided to utilise this experience and adapt it to this new field of application.

To improve and adapt it to transgenic projects, barrier procedures such as access to the unit, personnel clothing and protection were upgraded relative to the working environment.
These changes were introduced to improve the global biosecurity level, considering the number of lines / projects per unit, the experimental and monetary value of the transgenic lines and the labour intensity of the work (duration and type of handling / operations).

When used properly, what is the expected benefit of this housing system?

Considering the SOPF health status:

- to decrease experimental interference due to opportunistic agents;
- to improve the breeding performances; and
- to increase the life expectation (both for breeding and for long term experiments).

Considering the containment system :

- access to large scale housing capacity for a lower cost (both for capital expenditure and running costs);
- user-friendly working conditions for labour intensive projects; and
- more flexibility in the project management.

Ventilated filter top cages (or individually ventilated cages)

Our laboratories have developed a ventilated cage rack which has been validated in our transgenic department (technical and validation dossier available upon request). This system can now be used for specific projects complementary to isolators, ventilated cabinets and static filter top cages. The benefits of the active ventilation (lower ammonia, carbon dioxide and relative humidity levels) should be balanced against the significantly increased investment and running costs, their time consuming use and lower practical experience (for bioexclusion and mainly biocontainment overall efficiency). It appears that all these systems are much more complementary than alternative, hence covering a wide range of technical solutions to various situations.

Health monitoring of filter top cages

Each filter top cage is actually an independent microbiological unit. In consequence, a specific health monitoring scheme was developed for this application, based on three types of screening (full and reduced scheme and opportunistic microorgansms) carried out weekly, monthly and quarterly. (Please refer to Table 1).

Transgenic rodent holding : regulatory and biosafety issues

European regulations which govern the contained housing and use of genetically modified organisms (GMOs) for research purposes are :

- European Directive 90/219/EC 98/81/EC : contained use of GMOs/GMMs for research purposes
- European Directive 90/220/EC : deliberate release or commercial use / placing on the market of GMOs

Concerning GMOs, four classes of risk for personnel and the environment have been identified, with related containment levels and specific administrative requirements. For each GMO production and utilisation step, the global risk level will be estimated by taking into account the transgenic construct (donor organism + vector + receptor organism) including intrinsic characteristics and danger and the nature of each manipulation step.

For both Directives, each breeding or research establishment has to notify the national authorities and, prior to being approved / registered, has to fulfil legal requirements such as responsibility of named competent and authorised persons in registered / approved facilities, suitably equipped and submitted to inspection by a national authority, for a determined range of activity.

According to the classification of a transgenic line in accordance with these regulations, it is also compulsory to adapt the biocontainment level to the class of risk, in addition to the biosecurity and bioexclusion management issues already addressed.

Primary containment (protection of operators by biosafety cabinets class two and three) and secondary containment (protection of the environment using A2 - A3 biocontainment animal facilities or L2 – L3 containment laboratories) techniques and procedures are usually associated to meet the global specific experimental situations.

In addition to these biocontainment techniques, there is a need to address biosecurity in animal facilities (biocontainment of animals contaminated by murine pathogens and / or bioexclusion of VAF / SPF animals, immunocompromised or not).

National laws deriving from the European Directives and their implementation in each country of the European Union (EU) may differ significantly.

Genetic testing and applications

The main DNA-based tests are:

- DNA profile characterisation (RFLP / minisatellites);
- DNA amplification : standard PCR and TaqMan PCR (end-point, real-time);
- Southern blot; and
- Slot blot.

Their applications are:

- genetic monitoring of inbred strains and outbred stocks;
- strain / stock characterisation and differentiation;
- marker-assisted accelerated backcrossing (or “speed congenics”);
- estimation of genetic variation within a population; and
- parentage verification.

Examples

Transgenic lines and induced mutations (knockout)

A comprehensive set of DNA-based tests is available to facilitate the periodic monitoring of both transgenic and knockout strains of laboratory animals. The ability to transfer and implement existing protocols is critical to relieve a research laboratory of the tedious task of genetic monitoring.
This expertise also has to include the improvement of existing assays and / or the development of new assays to meet the investigators’ specifications.

DNA amplification

DNA amplification can be used to determine the carrier state of transgenic or mutant rodents and the carrier state or the zygocity of individuals from knockout / mutant strains.

In some cases it is possible to detect all of the three possible genotypes in a single reaction, homozygous, heterozygous and wild type individuals exhibiting one or two electrophoresis bands.

When compared with standard PCR, the TaqMan PCR presents several additional advantages :

- higher specificity and increased sensitivity (primers and probe specificity, utilisation of hotstart and UNG/uracil amplicon destruction) ;
- decreased post-PCR processing time and risk of DNA contamination (no opening of the reaction vessel) ; and
- potential to develop quantitative analysis.

Southern blot assay

A Southern blot assay can also be used to determine the genotypes from knockout mice. Again, homozygotes, heterozygotes and wild type individuals display different bands. A lane labelled MWSS contains Molecular Weight Sizing Standard bands.

In some cases, this technique can also be used to evaluate the number of integration sites of a transgene.

Slot blot assay

A Slot blot test can be used to determine the presence or absence of a transgene in a strain. Dark spots represent carrier individuals, absent or faint spots represent wild types.

For some protocols, homo- vs heterozygocity can be determined via optical density measurements of the spots.

Table 1. Comparison of isolator and filter top cage system (static)

ACCORDING TO EXPERIENCE AND USE IN IFFA CREDO, 1993 TO 1999	**ISOLATOR**	**FTC SYSTEM**
Capacity limitation	Yes	No
Experimental procedures	Difficult	Easy
Fixed and variable costs	High	Low
Capital expenditure	High	Low
Adaptability	Low	High
Maintenance of SOPF status	Yes	Yes
Containment of pathogens	Yes	No
Staff/unit management	Easy	Complex
Health monitoring method	Easy	Complex

Table 2. Practical advantages and disadvantages of various techniques

TEST	**ADVANTAGES**	**DISADVANTAGES**
Southern blot	Generally unequivocal results Useful for transgene and knockout Potential to determine zygocity	Time consuming Costly Use of radioactivity (not always). Requires more DNA
Slot blot	Faster than Southern blot	More time consuming than PCR

	Analyses large number of samples at one time	Use of radioactivity Not useable for zygocity testing
Standard PCR	Very fast Less expensive No need for radioactivity Generally non-equivocal results Potential to determine zygocity Analyses large number of samples at one time Very small amount of DNA can be used	Need more information to implement Contamination problems more likely than other techniques
Taq-man PCR	Higher specificity than standard PCR and increased sensitivity (utilises primers + probe, "Hot Start" and UNG / uracil Amplicon destruction) Very quick post-PCR processing Decreased contamination risk (no opening of reaction vessels) Potential to develop quantitative assays	Need more information to implement Contamination problems more likely than other techniques, but less likely than with standard PCR

Microsatellites testing

This is used for routine authentification of inbred and F1 hybrid strains. In Charles River we have selected 16 murine microsatellites PCR primer sets (eight for mice and eight for rats), which survey eight independent genetic loci. If research and development needs require, the development of species- or strain-specific microsatellites primers is possible.

DNA profile testing

DNA profile tests detect highly variable DNA sequence information which is more informative than isoenzyme analysis. These tests are also less labour intensive and thus less costly than cytogenetic or skin graft testing. Once a DNA profile has been established, the genetic identity of inbred and outbred animal strains can be assured through periodic monitoring.

This directed selection of preferred genotypes in transgenic rodents originating from non congenic / genetically standardised background lines allows the investigator to :

- reduce the number of backcross generations;
- reduce breeding time and cost; and
- produce research models faster.

(Please refer to Table 2).

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Session Three

Importation, quarantine and monitoring of laboratory animals, particularly rodents, for issue in Australia

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Introduction

The availability of laboratory animals which are disease and pathogen free and which do not have antibodies which are indicative of past exposure to agents of concern is critical to laboratory services. The course of acquisition from production facilities of known status, shipping under appropriate security and maintenance in housing of appropriate isolation presents its own demands on animals and users.

This paper will not deal with the logistics of handling, housing and airfreight of laboratory animals. Details of requirements are available from the International Air Transport Association (IATA) or from freight forwarding agents.

The number of specific genetic and standardised lines identified for particular tasks means that they are traded around the world in significant numbers. Disease control and welfare issues require particular housing arrangements, attention, and continued monitoring.

The nature of the laboratory animal, methods of housing and care and its relationship with humans create complexity in establishing disease freedom. This usually involves sample testing.

At the national and international levels there are obligations regarding animal health and welfare which affect this matter. Australia's traditional stringent quarantine arrangements which have successfully excluded many livestock diseases are also employed for laboratory animals.

Quarantine

Quarantine in Australia is the responsibility of the Australian Quarantine and Inspection Service (AQIS), an agency of the Australian Department of Agriculture, Fisheries and Forestry.

Quarantine requirements reflect the known disease status and value of such animals, methods of handling and the facilities in which they are held. They are designed to exclude exotic and zoonotic diseases and those troublesome in laboratory colonies. Current requirements centre on hantavirus, lymphocytic choriomeningitis virus, ectromelia virus and rabies but are mindful of other diseases.

Quarantine policy and legislation

Australia's policy has always been conservative but all quarantine restrictions have been scientifically based. Where there has been inadequate scientific information on which to base decisions the precautionary principle has been applied.

The Quarantine Act 1908, one of the early Acts of the new Commonwealth provides the legislative basis and authorises the making of Regulations (which specify how) and Proclamations (which specify what) regarding quarantine activities and commodities that may be imported. This subordinate legislation is made by the Executive Council (Governor-General and Ministers) but is scrutinised by Parliament. The legislation provides certain delegations and authorities to the Director of Quarantine who can authorise "protocols" or "conditions" which provide flexibility and detail necessary to address biological variation.

Australia has obligations arising from its membership of the World Trade Organisation. The Agreement on the Application of Sanitary and Phytosanitary Measures of the WTO mandates the use of risk analysis and, inter alia, consultation and transparency in quarantine decision making because of the potential for unjustified impediments to trade. The Agreement also mandates the International Animal Health Code of the Office International des Epizooties (OIE) - the world organisation for animal health as the standard for quarantine.

The Code provides the general basis for international movement of animals, their genetic material and products derived from them while minimising the spread of disease. The Code also provides the basis of inspection, quarantine and certification.

Australian quarantine policy and procedure were recently reviewed by a Quarantine Review Committee chaired by Professor Malcolm Nairn. The Committee recommended an Import Risk Analysis (IRA) process of routine risk analysis (in house) for common routine cases or where there were precedents and of non-routine risk analysis where there were not. The latter involves the formation of Risk Analysis Panels (RAP) of Experts. The mandated process involves consultation with stakeholders at various parts of the process, including the process itself, composition of the RAP and the draft IRA paper, so that it is truly transparent. The AQIS Bulletin advises interested parties of its intention to undertake an IRA, of the progress made and of the availability of the draft IRA.

Methods of risk analysis are addressed in the OIE Code. Much has been written on risk analysis, essentially a step by step evaluation of the risks of each identified disease from the colony of origin to release in the importing country, is done. This may be quantitative, semi-quantitative or qualitative. The Code addresses:

- country factors e.g., the disease status of the export country / facility;
- commodity factors i.e., the capacity of the animal or product to carry the disease(s);
- number of import units (to quantify the risk); and
- risk of domestic exposure (in the country / facility of import).

Risk management is the process by which the risks are addressed e.g. by testing or quarantine.

Quarantine requirements

AQIS has developed guidelines for the approval of countries to export animals and their products to Australia. Current conditions for the importation of laboratory rodents were introduced in December, 1998.

The AQIS permit does not absolve the importer from the necessity of obtaining permission to import under the Wildlife Protection Act 1983, should this be appropriate.

The conditions require the donor colony to be free from the following diseases or infectious agents during the 12 months prior to export:

- hantavirus;
- lymphocytic choriomeningitis virus;
- sendai virus;
- ectromelia virus; and
- rabies.

Importers may wish to test for other disease agents to protect their colonies.

The colony containing the animals for export must be housed in accommodation which precludes access by wildlife, including rodents, and be insect vector proof and free of ticks.

The animals to be exported and the donor colony must have remained clinically healthy and free from infectious and contagious diseases in the 30 days prior to export.

Each animal for export must be examined by an official veterinary officer during the 48 hours prior to loading and be fit to travel and free from evidence of infectious and contagious disease and external parasites. Specific Pathogen Free (SPF) animals are exempt from examination, but certification by an official veterinary officer and the veterinarian in charge of the donor colony of their SPF status must be provided.

Transport should be in a container as specified under IATA Live Animal Regulations. On arrival, all litter in the containers must be destroyed.

Imported animals

The imported rats and mice must be maintained in quarantine premises approved by the Chief Quarantine Officer (Animals). They must be kept in secure containers in a locked building with keys to be held by nominated responsible persons. No transfer of imported rats and mice or offspring is to be made without the permission of the Chief Quarantine Officer (Animals).

A register of all imported rats and mice must be kept by a nominated responsible person. This register shall contain the following information:

- source of animals;
- identity of animals;
- numbers of animals imported, used, born, weaned, transferred, died; and
- cause of death.

All animals on the premises must be immediately identifiable as to their source and quarantine status. Microchips may be used. Advances are being made in national and international standards for microchips so that readers of all brands will be able to read all microchips and registers of microchips will be linked for ready access.

Imported animals may be isolated in microisolators. Non-imported animals may be placed in the quarantine room for use for breeding or as sentinels for immunocompromised imported animals. Animals in contact with imported animals will remain in quarantine until the imported animals are released from quarantine. Husbandry and handling practices, including traffic flows, must be of a standard which ensures the integrity of the quarantine status of the imported animals and thus reduces the likelihood of spread of disease.

If any of the imported or contact animals suffer from or are suspected of illness, or death is suspected to have been caused by hantavirus, lymphocytic choriomeningitis virus or ectromelia virus, AQIS must be notified immediately.

Imported animals must remain in approved quarantine premises during the entire period of their use in research. The imported animals must be kept physically isolated from all other animals in the facility not of the same quarantine status. Any animals (including sentinels) in the quarantine facility that come in contact with imported animals will assume the same quarantine status as the imported animals. Imported animals, must, at the end of their use be disposed of in a manner approved of by AQIS (e.g., autoclaving or incineration).

Release from quarantine

Progeny of imported rats and mice may be released from quarantine to institutions registered by their State or Territory (an institution holding animal ethics clearance) to hold rodents if the above conditions are met.

Except for *Mus musculus, Rattus rattus* and *Rattus norvegicus*, permission must be obtained from Environment Australia to transfer rats and mice and their offspring from quarantine premises to other premises.

Progeny of imported animals are eligible for release only if prescribed tests are performed on a statistically valid sample of all the rats and mice at least eight weeks of age and show freedom from the following disease agents:

- hantaan virus - enzyme linked immunosorbent assay (ELISA)
- lymphocytic choriomeningitis virus- ELISA
- ectromelia virus (mice only) - ELISA

Other test methods may be used with prior approval from AQIS.

Several options for sampling of the imported animals are provided:

- one off sampling of imported animals and their progeny in the quarantine room. Sample size must be sufficient to detect a 5% prevalence of infection at a 99% confidence level and no introductions within 30 days of blood collection;

- sampling the colony of imported animals and progeny in the quarantine room on a quarterly basis over the previous 12 months. Each sampling to detect a 30% prevalence at 99% confidence and no introductions within 120 days of release i.e., 30 days before third sampling;

- progeny maintained as a separate biological unit from the imported animals, each sampling to detect a 30% prevalence at 99% confidence and no introductions within 120 days of release i.e., 30 days before third sampling.

For the second and third options, provided the colony is on a quarterly testing program, progeny can be released after two negative quarterly tests at least 30 days after the last introduction.

If any of the animals test positive, the officer in charge of the colony must notify AQIS. No release will be allowed and AQIS will give instructions as to further investigations required or the disposal of the positive animals and those in contact.

Where immunocompromised mice are imported, sentinels may be used, under the following circumstances:

- sentinels (8 to 12 weeks of age, the same species as those in quarantine) must be placed in contact (in the same boxes) with the imported animals on arrival in quarantine for a minimum of 45 days but not more than 120 days prior to testing for the diseases listed above;

- the number of sentinels to be placed in contact with the colony is calculated from the number of animals in the colony prior to adding the sentinels to give 99% confidence of detecting disease if it is present at 5% prevalence. A few additional animals should be added to the colony.

A report containing the test methods, the name of the testing laboratory, and numbers of animals tested must be provided to AQIS before approval for release will be given. The purchasers of animals may require pre-transfer testing for other disease agents.

Premises

AQIS requires imported biologicals and laboratory animals to be kept in approved laboratories. There are requirements regarding location, equipment, waste control and record keeping which are controlled through quality assurance arrangements which may be based on HACCP principles. This method allows the management to develop quarantine security arrangements which best suit their facilities and operations. It might be said that the process is outcome orientated so that the individual method is less important than the result. This suits the wide nature and purpose of laboratories very well and lightens intervention into regulatory measures while allowing the disease control and welfare needs to be met. Where necessary premises and records are audited.

Disease control

Disease control tends to be based on quarantine and the procurement of healthy stock from accredited or reputable sources. Transport in isolators and quarantine or isolation on arrival may be required. Design of housing or vivaria is important in prevention of cross infection. Bacterial diseases may be treated by chemotherapeutics in feed or water. Mycotic diseases are controlled principally by careful purchase and by control of the animal house environment.

Cleaning and sterilisation of equipment is important as are the sources of bedding and feed.

Biosecurity

Some animals will be SPF and will have to be moved and held in appropriate isolators to prevent introduction of organisms from which they are free. There are important zoonoses which require high levels of security. Biosecurity levels have been described by Murray (1998) and are recorded in the International Animal Health Code of the OIE.

Systems of biosecurity have four levels. The OIE Code specifies:

- Group 1 animal pathogens: enzootic disease organisms. No official control.
- Group 2 animal pathogens: exotic or enzootic organisms. Low risk of spread. Official control. Not vectored, species specific, limited economic significance.
- Group 3 animal pathogens: exotic or enzootic organisms. Moderate risk of spread. Official control. May be vectored, quarantine applied, severe economic significance.
- Group 4 animal pathogens; Exotic or enzootic organisms. High risk of spread. Official control. May be vectored, quarantine applied, movement controls, severe economic significance.

Laboratories handling groups 3 and 4 operate at negative pressure. compared to the environment, have HEPA filtration of exhaust air and treatment of liquid and solid effluent to inactivate organisms. Operatives must shower out. Group 4 also requires full isolation in closed (class 3) biosafety cabinets or the use of full body suits.

Disease concerns

The principal diseases in laboratory mice and rats that are of concern to AQIS include:

Ectromelia (mousepox).
Ectromelia is a highly contagious poxvirus infection of laboratory mice. Infection of naive mice can result in 100% of animals of susceptible strains (eg. BALB/c, C3H) affected. Animals may die in the viraemic stage or develop generalised skin rash. Sub-clinical ectromelia infection may be converted to clinical disease by many common laboratory manipulations.

Lymphocytic choriomeningitis virus (LCM)
LCM is a natural infection of wild and laboratory mice and Syrian hampsters. Humans, monkeys, dogs, rabbits, guinea pigs, rats and chickens are susceptible to infection and it is because of its zoonotic potential that imported animals have to be free of infection. Clinical disease in mice is highly variable depending on the virus strain, mouse strain and age at infection. In humans infection can cause serious and fatal disease.

Hantaan virus infection.
Hantaan virus is the prototype virus of a group of viruses in the Hantavirus genus. Hantaan virus causes clinically inapparent infection in rats but severe disease (Korean haemorrhagic fever) in humans. Naturally infected laboratory rats have been the source of hantavirus infection in research workers in Japan, Belgium, UK and France.

Other agents that are of less concern to quarantine authorities but which should be of concern to importers are:

Murine parvoviruses (MVM, MPV)
Mouse hepatitis virus
Pneumonia virus of mice
Reovirus type 3
Sendai virus
Theiler's encephalomyelitis virus
Rat parvoviruses (KRV, Toolan's H-1, RPV)
Mouse adenovirus
Rat coronaviruses (RCV, SDAV)
Mouse polyoma virus
Rotavirus (EDIM)
Mouse cytomegalovirus
Mycoplasma pulmonis
CAR bacillus
Clostridium piliformé
Encephalitozoon cuniculi
Citobacter rodentium (freundii BT4280)
Salmonella spp
Dermatophytes (ringworm)
Internal nematodes (eg., pinworm)
Skin mites

Conclusion

The AQIS processes of establishment and regulation of importation requirements are designed to protect the community and the animals concerned. They are scientifically based and must meet national and international obligations. The processes are transparent and stakeholders and interested parties have an opportunity to participate and should do so in order to ensure the best means of achieving disease control objectives are employed. Modern methods of self regulation through quality assurance measures are encouraged.

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Session Four

New infectious agents of rodents

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Abstract

This decade has seen the emergence of a number of "new" infectious agents of rodents, most notably MPV (mouse parvovirus) of mice, RPV (rat parvovirus) of rats (formerly the "orphan parvoviruses"), and helicobacter species, which affect both mice and rats. The availability of ever more powerful molecular diagnostic techniques and the increased use of immunocompromsied, transgenic, and aged rodents along with generally rising rodent censuses practically guarantee that this trend of emergence will continue. Some degree of serendipity always seems to be involved in scientific discovery; historically, this has been particularly true for infectious disease agents. Fortunately, today's laboratory animal diagnostician has an array of techniques to apply to the problem of detecting and characterising previously undescribed rodent pathogens. These techniques, some of which are remarkably "low tech" and simple to the point where they might be overlooked in the quest to use more sophisticated methods, vary greatly in sensitivity and specificity. However, these differences, especially those of specificity, can, as happened in the case of the "orphan parvoviruses", offer critical clues about the nature of these agents. Because novel agents are always first detected when an alert individual recognises the unusual or the unexpected, those of us involved in this endeavour should work just as hard on our powers of observation and communication as we do on our scientific skills. Close observation of animals and animal-derived experimental materials; recognition of odd, unexplained or contradictory diagnostic testing results or trends; and, perhaps most importantly, maintaining a good rapport with researchers and animal caretakers are all critical skills for detecting emerging agents.

Introduction

Dr Steffen opened by emphasising the importance of diagnostic laboratories in identifying emerging diseases and their causative organisms. He asks - is a "new" infectious agent one that has just come onto the scene which wasn't there before, or has it just been recognised because the investigator is more diligent? It is now possible to recognise a new agent due to polymerase chain reaction technology (PCR).

This paper addresses the discovery processes and includes four specific examples that have emerged in the last decade. Probably the newest and most significant rodent infectious agents to emerge in this time are the group of viruses previously known as the orphan parvoviruses. The other really interesting rodent agent discovered in the past six or seven years has been the discovery of helicobacter species of bacteria.

Mouse parvovirus

Inexplicable serology results from RADIL in the mid to late 1980s led to the suspicion of a new virus, even though attempts at its propagation were unsuccessful. McKisic *et al.*, (1993) reported on non-MBM parvovirus in a CD8+ T- cell clone cell line. Retrospective studies at Yale University later showed that this agent had been present

of the past 20 years. In other words, the "new" agent simply hadn't been detected until more sophisticated techniques were available.

The type strain identified by McKisic *et al.,* (1993) is now know as MPV-la (also known as MOPV, MPV or MPV-1), as there are other strains of mouse parvovirus known. MPV-la is now routinely detected by one of the following tests:

- rNS-1 ELISA / MPV HAI (RADIL); or

- rNS-1 ELISA with parvovirus-specific IFAs (IFA = immunofluorescent antigen)'; or

- parvovirus-specific IFAs.

Other techniques are used to differentiate which parvovirus is causing infection. The diagnosis is confirmed by the use of generic as well as specific PCR tests.

Rat parvovirus

This was detected at Yale University in control rats in a KRV study in 1995. The key once again was unusual serology results (positive IFA and negative HAI). There is as yet no specific test for this agent, which has proved difficult to grow.

There is also a hamster parvovirus (HAPV), first reported at RADIL by Gibson in 1983.

Helicobacter

Helicobacter hepaticus was first discovered in 1992, although this was not published until 1994. The original study used A / J Cr mice on a long-term toxicology study at the National Cancer Institute. It was noted that a number of these animals had chronic, active hepatitis and a marked increase in hepatocellular neoplasia. During a routine follow-up, H and E stains revealed a helically shaped bacterium. Soon after it was shown that liver homogenates from the mice transmitted the disease. Culturing this agent is quite difficult and it is unusual. It grows under *Campylobacter* - like promoting conditions and is slow-growing, producing a film-like swarming colony. It is easily missed when an agar plate is inspected. It can now be cultured from caecal tissue or faeces.

There is now a serologic technique which detects *H. hepaticus* (see Steffen *et al.,* 1995) . It is a membrane digest IgG ELISA developed by RADIL, which has a sensitivity and a specificity of >90%. This can detect infection by this organism as early as two weeks. In the genus *Heliobacter* there are a lot of species emerging, and it is likely more will be found, some of which will be true enteric pathogens. See Table 1 for a list of known rodent *Helicobacter* species.

Hyperkeratosis or "scaly skin disease" of nude mice

While this had been seen since 1976, its elucidation as a disease moved very slowly. It was first described by Clifford *et al.,* (1995), who described a sporadic and low incidence of scaly dry skin in nude mice. Even though they ascribed the cause to a unique *Corynebacterium* species, after "partially fulfilling" the Koch-Henle postulates, they were still unable to define its species. There may be environmental and hormonal factors contributing to its colonisation of nude mice and to the incidence of

clinical disease. It is interesting that Tichter *et al.*, (1990) described what was apparently the same syndrome, but reported the aetiologic agent to be *C. pseudodiphtheriticum* (D2). Biochemically, these two organisms can be fairly easily differentiated, so it appears there are two different potential aetiological agents here.

Table 1 **Rodent *Helicobacter* species(** from Fox and Lee, 1997)

Species	Host	Primary Site
mustelae	ferret, mink	stomach
hepaticus	mouse	intestine
bilis	mouse	intestine
rodentium	mouse	intestine
trogontum	rat	intestine
muridarum	mouse, rat	intestine
cinaedi	hamster	intestine
cholecystus	hamster	liver

Problems illustrative of new agents posed by this bacterium include:

- a low incidence of clinical disease with high colonisation rates (other contributing factors may be non-infectious, hormonal or environmental).

- discrepancies in identification of the aetiologic agent (a number of species of *Corynebacteria* are normal skin flora).

- the organism has also been found in haired mice.

- similar organisms have been found in clinically normal nude mice.

Rat respiratory virus (RRV)

What has been found over the last two years is that this is presumed to be an enveloped virus (as it is chloroform sensitive). The Koch-Henle postulates have not yet been fulfilled. Lesions are seen in 6-18 week old rats. It may also produce less serious lesions in mice and has an extremely high incidence in rat colonies in the USA. There is as yet no diagnostic test for this organism.

Histopathology has been important in RRV identification. Lung lesions comprise small, raised grey to white foci; paravascular lymphoid cuffs, as well as parenchymal lesions. While the bronchioles are not involved, there is intestinal pneumonia, characterised by infiltration of lymphocytes and macrophages, with occasional neutrophils.

Aetiology

The search for an aetiological agent involved the following possible causes;

- foreign body
- bacteria
- fungi
- viruses.

These were progressively eliminated, by the following methods:

i) *Foreign body aetiology* - negative result on histological evaluation by polarised light for bio-refringement material.

ii) *Bacterial aetiology*

Histology with special stains	**Result**
Modified Steiner's silver stain	Negative
Tissue gram stain	Negative
Acid fast stain	Negative

Culture	**Result**
Blood and chocolate agar	Negative
Mycoplasma agar	Negative
Lowenstein-Jensen (*Mycobacteria)*	Negative

PCR	**Result**
Mycoplsma	Negative
CAR bacillus	Negative
Universal bacterial primers	Negative

iii) *Fungal aetiology*

Histology with special stains	**Result**
Silver stains	
- *Pneumocystis carinii*	Negative
- Fungi	Negative

PCR	**Result**
Pneumocystis carinii	Negative

iv) *Viral aetiology*

Serology	**Result**
All known rodent viruses	Negative
Human respiratory syncytial virus	Negative
Bovine respiratory syncytial virus	Negative

Viral culture	**Result**
Rat pancreatic line (ARIP)	Cytopathic effect *
Mouse rectal carcinoma line (CMT)	Cytopathic effect *
Other mammalian cell lines	Negative
Embryonated chicken eggs	Negative

* Titers are low ($TCID_{50} = 10^4$)

IFA with *in vitro* propagated agent	**Result**
Affected rats	Positive
Normal rats	Negative

Electron microscopy	**Result**
Affected lung tissue	Negative
Affected pancreatic cell line	Negative
Affected CMT cell line	Negative

Biochemical characterisation	**Result**
Chloroform treatment	Sensitive

Experimental infection studies
In vitro propagated organism Not completed

At this point there is strong evidence that it does have a viral aetiology and no evidence that it has any other type of aetiology. RADIL is a large diagnostic laboratory, with a diverse clientele from universities, research institutes, biotechnology companies and laboratory rodent producers. Out of 150 routine submissions, our laboratory found a prevalence rate of 22.5%. This is very high and suggests that it is ubiquitous in North America.

Summary of detection of new agents

Detection of new agents is often by low technology methods, e.g., routine screening of animals on a long-term study revealed *Helicobacter sp.* The key was the use of the proper stain and observant pathologists. With RRV, the key was again an histopathological finding that was not explained by any kind of viral serology or by any other known aetiology. Scaly skin syndrome was simply found by observation of animals.

There are potential roles for:

- clinical signs / observation of rodents;
- serology;
- histopathology;
- classical culture (benchtop microbiology);
- unanticipated experimental results;
- viral propagation / cell culture; and
- molecular technique.

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