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CHAPTER

11

Medical Management and Diagnostic Approaches

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I. INTRODUCTION

This chapter reviews the basic principles of medical management of rat colonies and diagnostic approaches to detect infectious diseases of rats. As is the case with other species, rats are susceptible to a variety of injuries and diseases that can cause distress, morbidity, or mortality. Any facility that houses rats must develop monitoring programs designed to rapidly identify health-related problems, so they can be communicated to appropriately qualified veterinary or animal care personnel to be resolved. These programs generally consist of multiple components, some of which are directed toward individual animals, and others that assess the health status of rat populations as a whole. Relevant aspects of medical management of rat colonies include (1) individual animal monitoring for signs of illness and distress, (2) colony monitoring for morbidity and/or mortality trends, (3) microbiological monitoring of sentinel animals and/or the environment for the presence of monitored or excluded agents, (4) quarantine of incoming animals of unproven status, (5) screening of biological research materials brought into the colony, (6) diagnostic support for microbiological culture, serology, molecular diagnostics, necropsy, and histopathology, and (7) management of disease outbreaks.

II. INDIVIDUAL ANIMAL MONITORING AND CARE

A. Observation and Examination

Daily direct observation of rats for signs of abnormality is important from a humane and ethical standpoint (National Research Council, 2011), and is vital to quickly identify problems caused by mechanical failure, trauma, pathogenic organisms, spontaneous disease, or research manipulations (National Research Council, 1996). The persons given the responsibility for this observation need to have had sufficient training or previous experience with rats to adequately detect abnormalities that might be present. Grossly visible trauma or lesions may not always be present in ill rats, but subtler behavioral clues might be present. For this reason, it is important to be familiar with the normal range of behavioral patterns observed in healthy captive rats, which has been described (Saibaba et al., 1995). Methods in which rats are videotaped and later evaluated for facial conformations that are correlated with pain or distress, i.e., the Rat Grimace Scale, have been used as a research tool (Sotocinal et al., 2011), and similar use of these facial clues might have utility for real-time evaluation of rats for signs of pain or distress (Leung et al., 2016). Other research tools such as automated activity tracking may also have utility for identifying illness-related abnormal

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behaviors, even in social groups in some innovative housing environments (Brenneis et al., 2017).

Using appropriately sized equipment and methods similar to those used for larger species, it is possible to perform a relatively complete physical exam upon rats when indicated (Sharp and LaRegina, 1998). The use of fabric or plastic restraint devices and/or protective gloves may be needed if the animal is fractious or the individual doing the examination is inexperienced, but most strains of rats are fairly amenable to gentle handling.

Careful observation and diagnostic evaluation are indicated for animals that show clinical signs when irradiated, exposed to corticosteroids or other immunosuppressive agents, or subjected to other types of significant stress, because latent infections might become symptomatic at these times. Likewise, genetically immunodeficient animals (such as *rnu/rnu* rats) may manifest signs of disease from agents that are clinically silent in immunocompetent animals housed in the same area. Although the overall health status of most institutional rat colonies is monitored by routine screening of asymptomatic animals, it is important to realize that daily individual animal observation can sometimes identify an "index case" of a newly introduced disease that has not yet been revealed via routine scheduled testing.

B. Signs of Illness and Distress

Abnormal physical findings in rodents are not always useful in localizing an illness to a specific organ system. A very common constellation of findings indicative of pain, distress, or illness is piloerection, decreased activity, an ungroomed appearance, and often a hunched posture (National Research Council, 1992). Chromodacryorrhea (red staining and crusting around the eyes) is an accumulation of porphyrin-containing secretions that is sometimes associated with illness as a result of increased production due to stress or disease as well as decreased self-grooming behavior due to distress. Weight loss is another nonspecific finding, but since weight determination is a simple, rapid, objective, and noninvasive technique, it is commonly used to assess the general health status of an animal placed under observation. It should be realized that stress is not always manifested as an absolute weight loss in a growing animal, so it may be necessary to take into account the normal weight gain of young rats to document a variation.

Table 11.1 describes signs of illness that can be seen in rats, along with possible diagnoses. This list is not meant to be an exhaustive summary, but it includes some of the more common clinical signs and suggests potential differential diagnoses.

C. Treatment of Disease

The majority of drugs administered to laboratory rats are provided prophylactically (for example, as part of perioperative care) or as a direct component of the research study. Because both the disease state and the use of xenobiotics (antibiotics, analgesics, antiinflammatory agents, etc.) can affect the physiology of animals in a way that is difficult to control within the experimental design and could invalidate a study (Lipman and Perkins, 2002), ill rats are often euthanized rather than treated. However, the situation surrounding the incident should be carefully considered to determine whether it is prudent to gather appropriate antemortem diagnostic samples and to submit the carcass for necropsy evaluation even if the animal is euthanized. Likewise, a process to monitor animal mortality records and to perform necropsy on animals whose death is suspicious is quite important, because in some cases such an evaluation can allow early detection of a problem that otherwise would reoccur and eventually affect a much larger group of animals.

In some situations, it certainly is useful to treat individual animals or larger groups if the animals are considered valuable to an ongoing study or are not being used to generate sensitive data. It is beyond the scope of this chapter to describe particular pharmaceutical dosages and treatment indications, but the reader can be directed elsewhere in this volume for disease-specific recommendations. Well-referenced and comprehensive formularies that include rat-specific drug dosages are also available, written for veterinarians in both the laboratory animal and "exotic" pet specialties (Carpenter and Marion, 2017; Hawk et al., 2005). When bloodwork is indicated for diagnosis of a rat health issue, small-volume sample collection and automated analyzers can provide useful data. However, dilution of samples should be done only when such methods have been validated, because dilution does not always lead to predictably proportional results (Johns et al., 2018; Moorhead et al., 2016).

III. COLONY HEALTH MANAGEMENT

A. Need for Monitoring

Despite the fact that some infectious agents (for example, virulent strains of rat coronavirus) can induce readily identifiable abnormal physical signs, most infectious agents encountered in laboratory rat populations cause only subclinical disease. Such conditions can only be detected and identified via sensitive and comprehensive testing protocols (National Research Council, 1991). Despite the lack of observable morbidity or mortality, these subclinical infections pose a

TABLE 11.1 Physical Findings.

Abnormality	Potential Diagnosis
Pale mucous membranes, extremities, or eyes	Anemia (if rat appears otherwise relatively normal)
	Excessive or too frequent blood collection
	Circulatory deficiency (if animal appears weak or depressed)
Alopecia with normal, intact skin	Physical abrasion from cage or feeder
	Self-grooming or barbering from cage mate
Alopecia with crusted, inflamed, or ulcerated skin	Ectoparasites or dermatophytes
	Bacterial opportunists such as *Staphylococcus aureus*
	Pruritic syndromes
Dermal or subcutaneous masses	Tumors of skin or mammary origin
	Lymphadenopathy
	Abscess, granuloma, cyst
Nodular deformity of ears	Auricular chondritis
"Red" or "bloody" tears	Chromodacryorrhea caused by Harderian gland secretions (can also be seen on front paws and over the back from grooming)
	Frequent clinical sign of coronaviral infection but can also be a nonspecific finding of illness or distress
Circumferential, annular constrictions on tail	Ringtail (generally seen in suckling animals under conditions of low humidity and a cool or poorly insulated environment)
Head tilt, circling or spinning when lifted by tail	Bacterial or mycoplasmal otitis interna/media
	Tumor or other space-occupying brain lesion
Hairless, swollen, or bleeding plantar lesions	"Sore hock" syndrome associated with large and/or aged rats kept on wire or mesh flooring
Salivation, weight loss, swollen oral tissues	Malocclusion
Fecal staining	Diarrheal enteritis
Dyspnea/rales/hyperventilation	*Mycoplasma pulmonis*, *Filobacterium rodentium* (formerly CAR bacillus), *Corynebacterium kutscheri*, or *Streptococcus pneumoniae* infection
	Overheating
Facial swellings	Parotid and/or submandibular salivary gland swelling from coronavirus infection
	Abscess of lymph nodes (lymphadenitis)
	Zymbal gland tumor at the base of the ear
Abdominal distension (pot-bellied appearance)	Ascites
	Intestinal distension from toxicity (chloral hydrate)
	Enteritis (possibly megaloileitis associated with Tyzzer's disease)
	Obesity
	Abdominal mass (tumor, abscess)
	Pregnancy
Excessively wet hair coat and/or bedding	Diabetic polyuria
	Leaking bottle or automatic water system
	Behavioral water wastage from "playing"
	Overheating
Eye lesions	Blepharospasm, corneal opacities, keratitis due to coronaviral infection
	Cataracts (aging lesion)

significant risk to the research conducted with affected animals because they can alter the background physiology of experimental subjects or cause variation and alteration in specific experimental responses. These adverse effects have been summarized in a number of reviews (National Research Council, 1991; Baker, 1998, 2003; Nicklas et al., 1999; Lipman and Perkins, 2002), and more recent studies continue to add to the list of potential adverse implications (Besselsen et al., 2008). Two of the physiologic processes that have specifically been shown to be altered by the presence of these infectious agents in rodents include immune function and neoplasia, which are quite relevant because immunology, transplantation, and cancer biology are three of the disciplines that have historically depended heavily upon the use of rats (Gill et al., 1989). As a result, one of the primary aims of a rat colony health-monitoring program is to document the presence or absence of particular infectious agents irrespective of any observable disease state. A term that is generally synonymous with colony health monitoring and is often used to convey this emphasis is *microbiologic monitoring* (Fujiwara and Wagner, 1994; Waggie et al., 1994).

Although there has been great progress in defining and improving the overall health status of laboratory rat colonies in recent decades, many of the agents of concern remain endemic in institutional colonies (National Research Council, 1991; Jacoby and Lindsey, 1998; Livingston and Riley, 2003; Carty, 2008; Pritchett-Corning et al., 2009; Marx et al., 2017), or may be introduced via human contact or feral rodent contamination. Significant risks of one or more of these agents being introduced still exist in contemporary colonies. Because a comprehensive colony health-monitoring program is so vital in protecting the validity and reproducibility of experimental research data, it must be given appropriate priority in terms of budget, personnel, and other resources. The policies and practices should be defined in written plans, and agreement with the principles set forth should be secured by the scientific and administrative leadership of the institution, as well as by the veterinary and animal care group.

B. Health Status Terminology

The terms *axenic* and *gnotobiotic* refer to animals that harbor no cultivatable organisms or have a completely defined microbiological flora, respectively (see Chapter 21, Gnotobiotics and the Microbiome); as a consequence, the health status of these animals regarding pathogenic or opportunistic agents is relatively easy to characterize. Terms that are less useful without detailed additional information include *specific pathogen free* (SPF) and *conventional*. In general use, SPF refers to animals that are (1) considered to be free of major pathogens and some or all opportunists, (2) maintained under housing and use conditions designed to protect this high-quality status by excluding infectious agents, and (3) monitored closely to assure that there is no undetected introduction of excluded agents. Conventional animals are usually considered to be those that originate from uncontrolled colonies that are not subjected to routine health monitoring, or those in which some degree of monitoring occurs but there is no action taken if infectious agents are found. However, these terms are not really descriptive or representative enough to use when assigning risk to animals proposed for introduction to monitored, disease-free animal facilities. For example, animals from an institution that experienced an outbreak of rat coronavirus and decided not to undertake the steps needed to eliminate the agent from the facility can still be considered to be "SPF", since by definition this status is only defined by the particular list of agents of which the animals are specifically free. The term *conventional* also suffers from some ambiguity, since it can be used to refer not only to animal health status but also to facility design. For example, a facility that allows direct staff entry into rat rooms without changing out of street clothes would be termed a conventional facility rather than a "barrier" in the purest sense, but if microisolators and high-efficiency particulate air-filtered changing hoods have been used to successfully institute pathogen exclusion at the cage level, the animals themselves might possess a high-quality health status that is far from "conventional." In practice, it may be more effective to communicate the specific panel of agents for which the animals have been tested rather than assigning generalized quality descriptions.

C. Colony Health Management Considerations

In contrast to a program designed to monitor individual animal health through the use of direct methods such as close observation and physical examination, a program created to monitor the overall health status of a colony population will often utilize more indirect methods. Routine testing of selected representative animals (even in the absence of any signs of illness or disease) can provide valuable information regarding the viral, parasitic, and bacterial agents that such animals are either currently harboring or have been exposed to in the past.

Risk analysis should be done by any institution planning on holding rats, a process that should involve a discussion of the relative costs and benefits of the various options available for routine health monitoring as well as quarantine isolation and testing. Although the available expertise of trained veterinarians, colony managers,

and other professionals must be utilized, the discussions should not completely exclude the primary research directors and institutional officials who are needed to support the program both financially and administratively. It is also important to establish good communication with those individuals utilizing the animals for research so that they can report abnormal physiological responses or other experimental variation. It is not uncommon for research personnel to identify a problem with rodent-derived experimental data that ultimately is found to be due to microbiological contamination (Small, 1986; McKisic et al., 1993).

Risk-based sampling strategies will take into account (1) the frequency of introduction of new animals, (2) the quality and reliability of the source of introduced animals, (3) the mode of transport, (4) the pattern of personnel traffic into and out of the room, (5) the frequency of animal transport into and out of the room as part of the research project, (6) the potential for cross-contamination from other rooms inherent in the facility design, (7) the housing system, (8) the facility design (e.g., barrier configurations), (9) the proportion of the animals that are irreplaceable, (10) the proportion that are immunocompromised due to genetic factors, chemotherapy, or experimental stress, (11) the prevalence of infectious pathogens within the animal facility among laboratory rats in general, and (12) the potential for introduction of pathogens through the use of biological materials. The continued development of genetically engineered rats adds additional factors to consider when assessing risks of infectious disease.

It is important to realize that the window of detection varies for different types of diagnostic tests, and this must be taken into account whenever vendor screening or quarantine test results are interpreted. For example, when an antibody detection method (serology) is performed upon arrival, it is often considered representative of the vendor's colony, while subsequent seroconversion evident in serum drawn 2–4 weeks after delivery may be an indication of exposure during transport or shortly after arrival at the user facility. However, tests that directly identify components of the agent, such as an antigen detection assay or polymerase chain reaction (PCR) testing could theoretically be positive upon arrival due to either a preexisting vendor problem or in-transit contamination.

D. Specific Components of Microbiological Monitoring

The primary goal of health monitoring is to detect the presence of an organism in at least one animal in the sample population, provided the organism is present. Equally as important, such testing is the means by which any of a panel of agents may be confirmed as not present. The components of a typical colony health monitoring program may include (1) periodic routine assessment of resident animals via random screening or targeted testing of dedicated sentinel animals, (2) the assessment of incoming animals through the use of vendor screening and/or quarantine testing, and (3) the assessment of biological materials destined for use in rat experiments (for example, to verify that cell lines used for tumor studies are not contaminated with pathogenic or opportunistic infectious agents). The fourth component that is becoming more common is to use PCR testing to test portions of the room environment, especially by evaluating ventilated rack exhaust air dust from filters or plenums. However, as stated earlier, there should be a comprehensive program for individual animal monitoring, which can sometimes identify index cases of diseases in the early stages of outbreaks that have not yet been detected by overall colony monitoring.

There is great variety in rodent health surveillance programs, and no two designs are usually identical (National Research Council, 1991). However, some authorities advocate a certain degree of standardization (Jacoby and Homberger, 1999), and there are regional organizations that provide detailed specific guidelines for institutions that wish to participate (Yamamoto et al., 2001; Mahler et al., 2014). Ultimately, health monitoring program design should cater to the needs of the institution. Consideration should also be given to needs of other institutions that could receive rats from the home institution (for example, the sharing of genetically engineered rats).

1. Random Testing of Resident Animals

Health monitoring is often performed on representative residents removed from the colony for specific testing. When selecting animals for screening, there are certain points to keep in mind. Animals to be sampled should be taken from rack and shelf locations spread throughout the room to maximize the possibility of detecting an isolated focus of contamination. If multiple stocks or strains are present, an attempt should be made to sample representative rats from each of these subcolonies. It is also desirable to test both young and old animals (avoiding geriatric animals) if they are available, since parasite burdens may be higher in the young (National Research Council, 1991), while the old would have had the best chance of seroconverting to agents that may have not yet affected younger animals. In a breeding colony, ideal choices might be retired breeders and surplus weanlings. Immunodeficient animals are a good choice for detecting parasites and bacterial contamination, since they may have a lowered resistance to such agents and as a result support higher burdens.

However, it is important to remember that serology will be subject to false-negative results if performed on an animal with a genetic or induced immunosuppression that may impair the antibody response. The purpose of health surveillance is generally not to accurately determine the specific *prevalence* of infection or disease in an area, but rather to accurately identify its *presence* by finding at least one positive animal in an endemic colony (National Research Council, 1991). The minimum number of animals from a population that need to be tested to identify one positive animal can be viewed as a statistical exercise in random sampling. Probability theory can provide the equation necessary to determine the sampling size needed, based on the assumption that one is dealing with an ideal population (i.e., 100 or more animals, where all animals have an equal opportunity for pathogen exposure) and calculated based on variables such as the prevalence of infection (often estimated at 30%) within the population and the degree of confidence required in the result (Clifford and Clifford, 2001). These equations have been used to prepare charts that have been published to assist in the selection of sample size (Small, 1984; DiGiacomo and Koepsell, 1986; National Research Council, 1991). For example, if an infectious agent affects 25% of the rats in a population, one would only need to test 15 randomly selected individuals to have a 99% probability of detecting the agent. These calculations have the most robust application when dealing with large populations of animals held under conditions that provide little or no barrier to cage-to-cage transmission (e.g., an open shoe box or suspended caging with no filter tops) because, under those circumstances, agents are fairly uniformly distributed and would be expected to have a prevalence of 30% or higher. Under those circumstances, even if a room holds 1000 rats, it would still only be necessary to sample 8 of them to be 95% sure that an agent is not present. Many vendor quality assurance programs are based on this type of calculation.

2. *Targeted Sentinel Programs*

Alternatives to a random sampling approach are needed because a large percentage of rats in contemporary research colonies are housed under circumstances that do not result in a uniform distribution of transmissible agents, due to the popularity and utility of cubicles (segregating fewer than 100 animals into functional groups) and/or systems that provide a barrier at the cage level, such as static microisolators or ventilated rack caging. These housing systems are beneficial in decreasing the likelihood of disease transmission, but they also make it harder to detect infectious agents based on random screening protocols because the agent distribution is not uniform and the prevalence of infection may be far below 30%. Another problem with random screening techniques is the impracticality of selecting and testing animals from active research colonies without disrupting the ongoing research. For this reason, it is common to place *sentinel* animals into a colony for the sole purpose of health status testing. These animals are not assigned to any particular study, and under ideal circumstances they will be exposed to the same agents as the *principal* animals actually being used for research or breeding. Because they exist solely for the screening program, sentinels can be bled, sampled, or removed for nonsurvival testing at the discretion of the colony management without interfering with ongoing experiments.

Sentinels should be immunocompetent young adult rats (6–8 weeks of age) (Koszdin and DiGiacomo, 2002). The use of aged rats should be avoided if possible, because these animals may be more prone to false-positive seroreactivity (Wagner et al., 1991). Selection of a particular stock or strain of rat to be used as sentinels will vary, and there is no single correct choice. Using the same stock and source as the principals that are being monitored may be ideal because it eliminates the additional risk of contamination that would occur if animals were imported from another source specifically to be used as sentinels (National Research Council, 1991). For closed breeding colonies, this can be done by setting aside some of the animals bred locally to be used as sentinels, and if animals are commercially obtained, extra animals can be ordered along with the principal shipment. However, this approach is not always practical in nonclosed colonies consisting of animals of varying backgrounds from multiple sources, so it is common for facilities to order separate animals from a reliable commercial source to be used specifically as sentinels. In this case, a readily available outbred line is often chosen for sentinel use, since they are inexpensive and will generally mount a robust antibody response. Inbred lines can also be used, but it is important to consider any strain-specific limitations of disease susceptibility or immune responses, since these may affect their utility as sentinels. Occasionally, sentinels will be chosen specifically so that they have a coat color that differs from the principal animals to minimize the possibility that they will be mistaken for experimental animals and inadvertently used in experiments or for breeding.

Sentinels should be placed in physical proximity to the principal animals they are associated with to ensure that they are exposed to equivalent environmental contamination. It is desirable to place them in a consistent spot on each rack so that husbandry and research staff can anticipate their location. If a single cage is used, it is customary to place it on the bottom shelf, since it is assumed that the concentration of aerosolized agents and particulate fomites will be highest near the floor. There are no firm guidelines for the relative

density of sentinels, but for logistical reasons at least one sentinel cage should be in place on each rack or in each cubicle. The placement of one sentinel cage on each side of a cage rack (holding ~25–36 cages) has historically worked well in most situations. Other approaches can be taken, such as allocating sentinel cages to each breeding or experimental subgroup, placing multiple cages on each rack to increase the theoretical sensitivity of the program, etc. Depending on the specific design of the program, each sentinel cage may contain either a single animal or a small group of rats. When multiple sentinels of the same age are kept in a cage, it is rarely useful to sample more than one at any time, since the microbiological status of cohabitating animals is generally uniform. The use of sentinels in pairs does offer two advantages in that the other sentinel in the cohort can be used to confirm positive results found in the rat initially submitted for testing, and this also gives sentinel animals an opportunity for social contact. However, use of pairs does increase the numbers of animals utilized so this should be considered as well.

In housing situations where filter-topped cages are being monitored, it is a common practice to remove the lids from the cages used to hold sentinels, effectively keeping them in "open" cages. This is done to increase the exposure of the sentinels to environmental contamination that might be transmitted by either true aerosols or small particulate fomites that are generated and dispersed within the room as part of routine rodent care and use. However, it should be noted that, in this type of situation, the subpopulation of rats with the highest cumulative risk of becoming infected with an agent (for example, the sentinel cages receiving a constant flow of dirty bedding) are *not* being held with the same degree of cage-level containment as the principal animals, and if they do become infected, the amount of environmental contamination and subsequent cross-contamination to other cages in the home room or elsewhere may be increased. Since other open sentinel cages in the room would be at highest risk for secondary transmission, it also may become more difficult to determine the point of origin of an agent within a room if sentinels are becoming infected, not from their assigned principal cages, but from other sentinel cages. This can essentially give a type of false-positive result (Weisbroth et al., 1998). During the sentinel program planning process, the benefit of a potential increase in the sensitivity of open-caged sentinels to detect an agent needs to be balanced against these potential adverse effects.

The process of routinely transferring *soiled* bedding from principal rodent cages into sentinel cages will increase the sensitivity of a monitoring program and can decrease the duration of sentinel exposure needed to detect endemic agents (Thigpen et al., 1989). The specific procedures utilized for the collection of soiled bedding and the transfer to sentinel cages vary widely as a result of the different types of cage/rack/hood configurations that are used and because they must integrate with the specific procedural methods being used for overall cage changing. However, to ensure that the transfer of bedding is having a net positive impact on colony health (by aiding in the detection of excluded agents) rather than a net negative impact (by increasing the cage-to-cage transmission between principal cages) this practice should be standardized and incorporated into both written procedural descriptions and employee training programs. It should be realized that dirty bedding transfer may not reliably transmit all agents of concern in rodent colonies. For example, studies in both mice and rats suggest that coronavirus infections may be relatively easy to detect, but the respiratory mucosa-associated *Filobacterium rodentium* (previously known as CAR bacillus) may be difficult to transmit via transferred bedding and fecal pellets (Dillehay et al., 1990; Artwohl et al., 1994; Cundiff et al., 1995; de Bruin et al., 2016).

The optimum time interval between the placement of sentinels and their screening is another factor that has not been definitively determined. The time it takes a sentinel to be exposed to endemic infection would be expected to vary depending on specifics such as (1) the relative density of sentinels, (2) the frequency of cage changing and soiled bedding transfer, (3) the percentage of principal cages that have bedding sampled at each change, (4) the caging system in place, (5) the prevalence and transmissibility of the infectious agent present, and (6) possibly the macroenvironmental characteristics of the room, such as relative humidity and ventilation. Once a sentinel is exposed, there will be an additional delay until the development of an immune response ascends to levels that can be detected by serologic means. Experimentally, it has been shown that sensitive antibody determination tests can identify seroconversion in a period as short as 1 week postexposure for rats infected with agents such as the rat coronavirus/sialodacryoadenitis virus (RCV/SDAV) (Smith, 1983) and the rat virus (RV) parvovirus (Ball-Goodrich et al., 2002). However, a more "average" timeframe is within the range of 2–3 weeks, and it is felt that the utility of testing results will be greatest if a period of 21–28 days is allowed for seroconversion. For this reason, sentinels should generally not be sampled before they have had at least 1 month of exposure. It cannot be assumed that an agent will make its way into a sentinel cage during the first week or two, so many programs allow for an exposure period longer than 1 month (for example, utilizing 2–3 months of exposure as part of a quarterly monitoring schedule). Of course, with the increasing use of PCR in screening programs, it is possible to directly assay for the presence of nucleic acids signifying the presence of viral,

bacterial, or parasitic contamination in samples such as fecal pellets, skin scrapings, or plucked hair.

3. *Vendor Screening*

Facilities wishing to verify the reports obtained from commercial colonies may establish formal vendor screening programs whereby a small group of rats are obtained specifically for diagnostic testing. Sampling of animals that are euthanized immediately upon arrival can provide confirmation of the health status of the animals as maintained by the vendor, although it should be recognized that serology would generally not be expected to consistently identify animals infected less than a week previously. PCR testing, bacterial culture, and direct examination for parasites can identify contamination over a shorter period of time, but there may still be a latent period. If the intent is to fully evaluate the status of all animals delivered, this testing must be repeated for each breeding unit of animals accepted from the vendor, and it should also account for the fact that vendors may produce the same strain in multiple, physically distinct breeding or holding areas (Small, 1984). Such a program might be feasible for facilities with a very limited list of vendors and a small number of strains in use, but is often impractical for facilities that serve large, multidisciplinary institutions. In this situation, a more limited and targeted vendor surveillance program might be useful (for example, surveying animals when a new vendor is under consideration, or getting more information if there are specific concerns about the status of animals from a particular vendor for some reason). Occasionally, the status of the vendor's production colony is not in question, but possible contamination during transport and delivery is suspected. If that is the case, incoming vendor animals for testing should not be killed upon arrival, but should be placed in a quarantine facility that provides for not only containment but also exclusion of infectious agents (to eliminate confounding cross-contamination within the facility). They can then be given time to fully colonize with and/or seroconvert to agents they were exposed to in transport, and tested on a schedule similar to other animals subjected to quarantine.

4. *Quarantine*

In many cases, the relative risk to the existing colony from newly acquired animals that are shipped from a high-quality vendor and arrive in intact, filtered shipping containers is small enough to allow direct introduction into the room (Small, 1986; National Research Council, 1996). The documented procedures for rodent receipt under these circumstances should include a careful inspection of the containers upon arrival, the rejection of those that are damaged, and careful handling and disinfection of the external surfaces to minimize the risks from superficial contamination of the crate.

In contrast, animals proposed for introduction from noncommercial sources are often bred, packed, and shipped under less stringent conditions, and the establishment of a quarantine program for this type of transfer is very important.

The type of health monitoring documentation available when animals are obtained from a university, research institute, or biotechnology/pharmaceutical company may be quite variable, and should be carefully interpreted as plans are made to receive and quarantine rats. Terms such as *SPF* or *conventional* are useful in relaying the general status of a colony, or to contrast the differing characteristics of animals from different rooms/buildings/facilities (much the same as the terms *clean* and *dirty*) but much more specific information should be obtained from the sending institution. From a health monitoring perspective, the status of each cohort of imported animals must be defined individually, based on the recent and historical findings of specific health monitoring tests. When introducing animals into a disease-free facility and making decisions about the relative risk, all animals should be considered suspect until there are data to suggest otherwise.

As is the case with mouse quarantine programs, there are a variety of ways to structure and schedule the collection of diagnostic samples. Historically, quarantine testing often resulted in prolonged delays of 8–12 weeks from the time of arrival until the time of release, but by utilizing PCR testing as an added component it is now possible for comprehensive testing to be completed in less than a month. The minimum list of agents evaluated during quarantine should be driven by the list of agents monitored or excluded in the area where the animals are destined to be used, but it is certainly acceptable to expand the list to perform a more comprehensive screening.

It is vitally important to achieve functional segregation and isolation of animals during a quarantine period, not only to protect the health status of other rodents in the facility, but also to ensure the ability to accurately determine the actual source of any contamination identified during quarantine. Room-level isolation would be ideal, but often there are space constraints when dealing with small shipments of rodents, and the common procedure is to utilize flexible-film isolators, cubicles, or ventilated cabinets of some type to partition a quarantine room (Small, 1984). In contemporary colonies, the introduction of a novel, noncommercial rat strain is a much less frequent occurrence than the transfer of a mutant mouse line. However, if this activity increases in the future, it may be necessary to consider programs similar to those described for mouse quarantine that group multiple shipments into a single cohort for batch

testing (Rehg and Toth, 1998). The availability of microisolator-type caging, either as static units or within ventilated racks, has also allowed programs to be designed that are not all-in-all-out but still allow functional isolation and segregation of multiple shipments within the same room (National Research Council, 1996; Otto and Tolwani, 2002). Although this option will provide more flexibility and may reduce the space requirements for quarantine, appropriate operational procedures are extremely important, since the whole system is reliant upon proper technique.

5. *Screening of Imported Biological Materials*

All tissue cultures and tumors should be tested and approved as free of infective contaminants prior to use in rats (Sharp and LaRegina, 1998; Peterson, 2008). Parvoviruses such as KRV and H-1 are examples of rat viruses known to contaminate cell lines, and the fact that H-1 was originally isolated from a human cell line after it was passaged through rats (National Research Council, 1991) provides a rationale for testing human cell lines unless it has been verified that they have no history of rodent passage. Recent experiences have shown that even cell-free biologicals have the potential to introduce agents to rodent colonies when imported (Lipman et al., 2000). Similar to the procedures used for mouse tissues, a rat antibody production (RAP) bioassay can be performed, whereby naïve animals held in quarantine are inoculated with a representative aliquot of the suspect material and tested 4–6 weeks later for seroconversion to excluded agents (Small, 1984; Johnson, 1986). However, PCR technology has supplanted mouse antibody production/RAP assays and allows direct testing of the materials themselves for the presence of infectious agents (Bauer et al., 2004; Bootz and Sieber, 2002; Blank et al., 2004).

IV. DIAGNOSTIC TESTING

A. Agents to Be Monitored

Over the past several decades great strides have been made in the identification and eradication of infectious agents from laboratory rodents. As a result, most contemporary biomedical research rat colonies are relatively free of pathogenic viruses, parasites, bacteria, and fungi that cause clinical disease. However, some microbes, especially those agents that cause latent or subclinical infections, remain a biosecurity risk to research rodent colonies (Carty, 2008; Besselsen et al., 2008; Cagliada et al., 2010; Mahler and Kohl, 2009). Many of these agents, independent of their pathogenic potential, have an impact on physiologic parameters of the host and may confound scientific results, increase experimental variability, and necessitate the use of a greater number of animals.

Timely and accurate diagnosis of adventitious infectious disease in rodent colonies is critical to the success of biomedical research. To this end, institutional veterinarians must closely monitor the health of research rodents through periodic and systematic examination of sample groups of research and sentinel animals against a predetermined list of infectious agents. The exclusion of an agent should be justified based on the potential for an adverse effect on animal health or research studies and must include considerations for host species specificity, zoonotic potential, prevalence, and host immune status (National Research Council, 1991; Mahler et al., 2014). There is no broad consensus on infectious agents that should be excluded from high-quality rat populations, but there is objective data documenting the agents that have the greatest potential for detrimental impact on research and thus are almost universally monitored for and excluded (Waggie et al., 1994; National Research Council, 1996; Nicklas et al., 2002; Baker, 1998, 2003; Besselsen et al., 2008). These agents are listed in Table 11.2.

There are a number of agents not on this list that also have the potential for significant impact on the host and merit monitoring in rat colonies. *F. rodentium* (formerly

TABLE 11.2 Core Agents for Screening.

Type of Organism	Specific Agent (Abbreviation)
Viruses	Kilham rat virus (RV or KRV)
	Toolan's H-1 parvovirus (H-1)
	Rat parvovirus (RPV)
	Rat minute virus (RMV)
	Pneumonia virus of mice (PVM)
	Rat coronavirus (RCV or SDAV)
	Rat polyomavirus 2 (RatPyV2)[a]
	Sendai virus
Bacteria	*Clostridium piliforme*
	Helicobacter spp.
	Mycoplasma pulmonis
	Rodentibacter pneumotropicus[b]
	Rodentibacter heylii
	Rodentibacter ratti
	Streptococcus pneumonia
Parasites	*Radfordia ensifera*
	Syphacia muris
Fungus	*Pneumocystis carinii*[a]

[a] *Clinical and/or histologic disease is primarily in immune-compromised rats.*
[b] *Formerly* Pasteurella pneumotropica.

cilia-associated respiratory bacillus) may be routinely monitored using serological and/or molecular assays (Franklin et al., 1999; Ike et al., 2016; Hook et al., 1998; Kawano et al., 2000). Although rarely encountered in rat colonies maintained at a high health status, some institutional veterinarians choose to monitor for Seoul virus and the bacterial agent *Streptobacillus moniliformis*, because they can cause a zoonotic disease and could be a marker for wild rat contamination (Bleich and Nicklas, 2008; Eisenberg et al., 2016a, 2016b; Firth et al., 2014; Cross et al., 2014; Kerins et al., 2018; Mc et al., 2017; McElhinney et al., 2016; Verner-Carlsson et al., 2015; Easterbrook et al., 2008). Other potential pathogens include reovirus, adenovirus, and rat polyomavirus 2 (RatPyV2). RatPyV2 is relatively prevalent in rats and has the potential to cause clinical and/or histologic disease primarily in immune-compromised rats (Besch-Williford et al., 2017; Masek-Hammerman et al., 2017; Rigatti et al., 2016).

The frequency of testing for a specific agent may be determined based on the perceived risks of infection, transmissibility, potential impact on the population and associated research, immune competence of the colony being screened, ubiquity of agent, and the requirements of the biomedical research community; however, it must be recognized that economic considerations also play a role (National Research Council, 1991). One approach to health monitoring is to group agents that pose a similar biosecurity risk based on prevalence in the population. For example, relatively prevalent agents such as coronavirus, parvoviruses, RatPyV2, rat theilovirus, *Pneumocystis carinii*, *Helicobacter* spp., *Rodentibacter pneumotropicus*, *Rodentibacter heylii*, *Rodentibacter ratti* (formerly *Pasteurella pneumotropica* biotype Jawetz, Heyl, and Taxon B), and pinworms might be tested for on a quarterly basis, while more infrequently detected agents such as Seoul virus, adenovirus, pneumonia virus of mice (PVM), reovirus, Sendai virus, *Salmonella* spp., and *S. moniliformis* could be surveyed on a semiannual or annual basis (Pritchett-Corning et al., 2009; Liang et al., 2009).

B. Tests Used in Health Monitoring

Optimal health monitoring programs for the detection of infectious disease in laboratory rats requires a multifaceted approach. Testing modalities may include gross necropsy, examination of serum for antibodies to infectious agents (serology), culture of bacterial pathogens, molecular evaluation of biological (feces, lung, pelt swab) and/or environmental samples by PCR designed to amplify infectious agent genomes, microscopic examination for parasites, and histologic examination of tissues. For routine health monitoring, these tests are often packaged, depending on the institution's needs, into profiles that include one or more testing modalities. For additional reading, a number of excellent reviews are available (Weisbroth et al., 1998; Compton and Riley, 2001; Feldman and Feldman, 2001; Livingston and Riley, 2003; de Bruin et al., 2016).

1. *General Test Performance Guidelines*

Determining the optimal test modality to use in detecting infectious disease requires some knowledge about the test performance, the host response to infection, and the natural life cycle of the organism. An ideal test is one that in all cases clearly distinguishes between infected and uninfected animals (Weisbroth et al., 1998). Diagnostic tests can be appraised via several parameters [Table 11.3 (Bellamy and Olexson, 2000)]. In general, diagnostic sensitivity and specificity are of greatest importance when designing a health monitoring program. Tests with high sensitivity will generate a very low percentage of false-negative results, whereas tests

TABLE 11.3 Parameters for Assessing Diagnostic Test Performance.

Test Characteristics	Formula
Diagnostic sensitivity—likelihood that an animal will be positive for a particular test, given that that animal is truly infected with the agent	$TP/(TP + FN) \times 100\%$
Diagnostic specificity—likelihood that an animal will be negative for a particular test, given that that animal is truly free of that agent	$TN/(FP + TN) \times 100\%$
Positive predictive value—estimate of the likelihood that an animal with a positive test has an infection; provides an estimate of the percentage of animals that are likely to have an infection, given that they are positive for a particular test	$TP/(TP + FP) \times 100\%$
Negative predicative value—estimate of the likelihood that an animal with a negative test is free of the infection; provides an estimate of the percentage of animals that are likely to be free of an infection, given that they are negative for a particular test	$TN/(TN + FN) \times 100\%$
Diagnostic accuracy—provides a measure of all results (positive and negative) that correctly classify infectious disease status	$(TP + TN)/(TP + FP + TN + FN) \times 100\%$
Prevalence—an estimate of the frequency of an infection in a population at a point in time	$(TP + FN)/(TP + FP + TN + FN) \times 100\%$

FN, False-negative results; *FP*, false-positive results; *TN*, total negative results; *TP*, total positive results.

with high specificity will generate a low percentage of false-positive results. Tests with high (>90%) sensitivity and specificity (for example, serology) should be used when available. For those tests that lack sensitivity or specificity (for example, histology), results must be interpreted accordingly. Other parameters, such as positive and negative predictive values, may also be of value in interpreting results; however, these parameters can be affected by agent prevalence. For example, when agent prevalence in a population is low, the calculated negative predictive value is high; conversely the positive predictive value may be misleadingly low (Lipman and Homberger, 2003). In other words, for an agent with low prevalence in the population, a negative test result confidently rules out the presence of disease, whereas a positive test result may be due to a false positive rather than a true positive. While highly sensitive and specific tests are available, it should be realized that no test is 100% sensitive or 100% specific. To this end, all unexpected results should be confirmed either using corroborative testing platforms, testing of cohort animals, or both. In no case should a decision about colony status be made based on a single positive result.

As discussed earlier, daily observation is a critical component to any health monitoring program. Recognition of clinical signs is especially important in the early detection of outbreaks of disease and documenting emerging diseases. However, because most agents that infect rats cause subclinical disease, observation is a very insensitive means of screening for infectious disease. As a result, sentinel and colony monitoring programs have been developed. For health monitoring, animals may either be euthanized and a necropsy examination performed, or samples may be collected from live animals such as blood for serology, feces for molecular diagnostics, swabs of the oral cavity for microbiological culture, or perianal tape test samples for pinworms.

2. *Testing Methodologies*

a. Necropsy

The term *necropsy* is derived from the Greek words *Nekros*, meaning corpse, and *Opsis*, meaning to view. The necropsy examination, including tissue collection and preservation, represents one of the most important phases for the evaluation of rats on study. Proper conduct of the necropsy is critical because there is only a single opportunity to conduct the procedure. Although there are many variations of necropsy technique, one should use systematic examination to ensure that all lesions and specified target tissues for collection are thoroughly examined macroscopically, as well as collected for subsequent experimental evaluation such as histopathologic analysis. The actual necropsy technique used depends on the objectives of the procedure. Most rat necropsies are conducted within the following categories: (1) diagnostic necropsy for determination of cause of clinical outcome, (2) rodent health surveillance, (3) complete necropsy for experimental purposes, and (4) target organ collection or evaluation. The methods used for these differing purposes will vary quite substantially and have been reviewed (Feldman and Seely, 1988). In many studies the necropsy procedure will need to be customized to suit the experimental needs.

Generally, the function of the gross necropsy is to identify lesions present in the animal and to collect tissue in an orderly fashion for subsequent microscopic and other experimental evaluations. Only the systematic necropsy technique can ensure that all lesions and specified tissues are examined macroscopically and collected for histologic and other analyses. Even the most careful microscopic preparation and examination cannot salvage tissues lost because of autolysis, tissue mishandling, or the failure to recognize and select lesions. A tissue discarded at necropsy is lost forever and with it potentially valuable information. Whenever possible, the rat necropsy should be guided by a standard operating procedure and conducted by trained prosectors. Although dissection of the rat and collection of tissues is often the goal, the quality of the necropsy depends on the entire procedure and highly depends on the degree of planning and preparation for the event (Table 11.4) (Black, 1986).

A properly conducted necropsy reduces the number of artifacts noted in tissues. Artifacts can be produced by a number of conditions, including excessive tissue manipulation, improper dissection technique, poor instrumentation, osmotically injurious moistening fluids, autolysis, and poor fixation. Excessive digital manipulation and improper use of instruments, such as the use of dull instruments, can easily lead to injury of fresh tissue with distortion of normal tissue architecture. This is particularly true with rats, in which small tissues are easily distorted by poor handling, such as crush injury resulting from improper scissor use. It is important to optimize dissection technique. For example, stretching the optic nerve during removal of the globe from the skull can lead to extensive artifacts such as retinal detachment. Artifacts can result from the drying of tissues or the contact of tissues with fluids that are not osmotically compatible. These changes can be reduced by the use of physiological saline as a moistening fluid and the avoidance of tissue contact with water. Artifacts of autolysis can be minimized by the proper use of fixatives and by ensuring rapid mucosal contact with fixatives. The need for rapid fixation means that it is important to flush rat nasal passages with fixative and to infuse fixative intraluminally into bowel segments.

Necropsy Preparation: The rat necropsy is a procedure that should be carefully planned to obtain the optimal

TABLE 11.4 Issues to Consider in Logistical Planning of the Rat Necropsy.

- What is the method of euthanasia, and is it compatible with experimental needs?
- What are expected "target organs," and are any special procedures warranted such as ancillary clinical pathology studies?
- What special prelabeling and identification requirements are needed?
- Do fixatives and/or clinical preservatives need to be prepared fresh?
- Are there randomization issues and/or special timing issues required between experimental groups?
- Are target organs to be weighed, and is exsanguination required?
- Do terminal clinical, clinical chemistry, urinalysis, or hematology specimens need to be collected and, if so, by what method? Is food restriction required before this collection?
- Are microbiology samples to be collected, and is an aseptic technique required?
- Are there critical steps that require a "single individual" to collect target tissues for consistency purposes?
- Is a special tissue collection order required for target organ study because of autolysis issues or biochemistry needs?
- Are special identification methods to be used for multiple or paired tissues?
- Are study protocols, standard operating procedures, and clinical records available to the prosectors?
- Are frozen specimens to be collected and, if so, by what method?
- Are morphometric techniques to be used, and does this require special tissue handling such as organ perfusion?
- Are there special postfixation needs for molecular, ultrastructural, histochemical, or immunohistochemical studies?
- What are the microbial, radiological, and chemical hazards posed by the necropsy procedures, and what precautions are to be used?

results. During the logistical phase of the necropsy planning, research personnel should review critical questions that will impact the conduct of the procedure and make plans accordingly (Table 11.4). The purpose of the necropsy should be carefully reviewed and will dictate the subsequent course of action. One must carefully consider the ancillary tests that will be performed on samples taken at necropsy, and this may dictate the order of collection and the necropsy methods that are chosen. For example, animal health and sentinel rat necropsies used to determine the pathogen status of colonies might necessitate aseptic technique and methods to acquire proper tissue samples for microbiological analysis. The specific types of organisms that might need to be cultured and the proper media to have available should also be considered in advance. Similarly, samples might need to be analyzed for the presence of macroscopically visible ecto- or endoparasites using special methods. There are many ancillary imaging and other specialized methods that might need to be used during a rat necropsy for experimental purposes, such as the use of radiographic methods for the detection of skeletal abnormalities.

If a large number of rats are to be subjected to complete necropsy using multiple prosectors and necropsy stations, one must determine whether single individuals should be responsible for certain technical aspects of the necropsy to maintain consistency of data. One such example would be tissue dissection for target organ weight determinations where trim methods might contribute to differences in outcome. There are situations in which individuals each having a particular necropsy task are preferable to the use of multiple stations in which each individual performs a complete necropsy. One must carefully determine the need for randomization of animals across study groups at the terminal sacrifice. Sometimes randomization is warranted; at other times it might be useful first to necropsy controls before experimental animals to allow easier determination of macroscopic observations. Whenever multiple prosectors are used, careful records need to be kept about who is responsible for individual prosections or individual organs. It is important to have individuals work across experimental groups to prevent operator-specific tissue handling artifacts from being confused with experimental outcomes. These types of decisions are best made during planning sessions before the actual conduct of the necropsy procedure.

Rats should be necropsied as soon as possible after death because postmortem changes begin to occur immediately. Microscopically, these autolytic changes closely mimic the histologic alterations that one might note in many types of acute experiments and have been well described (Seaman, 1987). If immediate necropsy is not possible, the rat should be refrigerated to slow autolysis. Carcasses should not be frozen if microscopic examination is to be performed because ice crystals cause disruption of cellular architecture.

Necropsy Equipment: All equipment and supplies should be arranged before the beginning of the necropsy procedure. Personnel should wear personal protective equipment, including laboratory coats, protective gloves, and safety eyewear. In addition, individuals need to be protected from volatile anesthetics, fixatives, solvents, and the like; thus chemical safety hoods, vented enclosures, downdraft necropsy tables, and scavenging devices are often present in rodent necropsy areas. Depending on the type of study, it might be necessary to have equipment such as biological safety cabinets and autoclaves present in the necropsy room to handle biological hazards. Modern rodent necropsy rooms are often equipped with computer stations for online collection of body/organ weight and necropsy data.

Prelabeling of equipment such as specimen containers, tissue cassettes, and weigh boats is useful to save time and to prevent sample mix-ups. All specimen containers should be labeled with indelible inks on their sides (never on container tops); this will help to avoid specimen mix-up. A cutting surface is necessary to lay the rat upon, and various hard plastic or cork dissecting boards have been used. Some prosectors favor corkboards or disposable or Styrofoam boards because they allow the use of pushpins to fasten rodents to the surface. In general, the surface should be easily cleaned between animals. If cork is used it must be covered with disposable paper, which is changed between animals, and the board must be thoroughly scrubbed at the end of the day. Lighting and magnification are important factors to consider during the design of the rat necropsy station because both tissues and lesions can be quite small.

Surgical instruments are satisfactory for the necropsy procedure, and instrumentation depends on personal preference but should include bone-cutting forceps or rongeurs to penetrate the rat skull, as well as clean, sharp, well-maintained scissors, forceps, and scalpels. Poor choice of instruments or instruments that are dull and soiled will cause tissue artifacts despite otherwise good dissection technique. An example includes large scissors, 15–18 cm in length, with sharp/blunt points that are suitable for the initial cutting through the skin and opening up the body cavities. For dissection of internal organs, various smaller, 10–13-cm long scissors, either straight or curved with blunt/sharp or sharp/sharp points, are adequate. Scalpels with no. 11 or no. 22 blades for trimming tissues are useful and may be used to prevent artifacts related to tissue compression from scissors. Various straight and curved forceps are used during the rodent necropsy, and "rat-toothed" forceps are helpful for gripping surrounding tissues, although great care must be used to prevent injury to target tissues. Surgical spatulas are helpful for lifting small organs such as the pituitary when forceps become impractical.

A variety of consumables and other supplies are needed for the rat necropsy procedure. Suture material or umbilical tape are used for ligation of tissues such as trachea/lungs and urinary bladder after infusion of fixative. Physiological saline is used to rinse tissues. Blunt-tip hypodermic needles/dosing needles and a 5–20-mL syringe are needed for fixative infusion. In addition, dyes for labeling lesions, gauze sponges, various bags, tubes, and containers are all necessary supplies to have readily available. Small tissues such as rat lymph nodes or adrenal glands should be placed into prelabeled tissue cassettes or embedding bags.

It is often important to weigh the carcass as well as target tissues and to have properly calibrated balances available for this purpose. In some experiments the brain is weighed to derive organ/brain weight ratios for assessment of parenchymal organ size. Body weight, although useful, can change with conditions such as obesity and make organ/body weight ratios misleading, although this parameter is commonly assessed in rodent studies. Rat organs and lesions are often measured, so calipers and other measuring devices such as metric rulers are useful to have available. A well-designed and located writing surface is useful to keep documents such as necropsy sheets away from the dissection work area.

Necropsy Procedure: There is no single best method for the conduct of the rat necropsy, and the method should be selected based on the objective of the experiment, use of the tissues to be sampled, and the experience of prosection and pathology personnel. The suggested background reading and references by the following should aid those that have an interest in the conduct of the rat necropsy (Greene, 1963; Cook, 1965; Olds and Olds, 1979; Wingerd and Stein, 1988; Popesko et al., 1992; Bono, 1994; Feinstein, 1994; Sharp and LaRegina, 1998; Bono et al., 2000; Parkinson et al., 2011; Fiette and Slaoui, 2011). The general necropsy procedure described in this section has proved useful in our laboratory.

b. External Examination

The first step in the rodent necropsy is a thorough external examination. The animal is palpated for gross anatomical abnormalities, morphologic asymmetry, or subcutaneous masses. The anus and urethral areas are examined for discharge, blood, or diarrhea. The oral cavity (Fig. 11.1A) is examined for malaligned or broken teeth, blood, or foreign objects (food or bedding). The ears and nose are examined for discharge. If identification implants such as ear tags or microchip transponders are used, their associated tissues are examined for the presence of macroscopic lesions. Eyes are examined for discharge and corneal or lens opacity. The external genitalia are examined for developmental abnormalities. The color and texture of the fur is also examined for any abnormality. Previous clinical observations are reviewed, and the animal is checked for any external finding to confirm observations, such as an injury. The animal's body weight and all external findings should be noted on a necropsy record form.

c. Exsanguination

Blood samples are often collected at the time of necropsy from the heart of the anesthetized rat via cardiac puncture, from the vena cava, or after decapitation from the body trunk. Even if blood samples are not required, it is desirable to completely exsanguinate the animal to obtain consistent organ weights, provide for

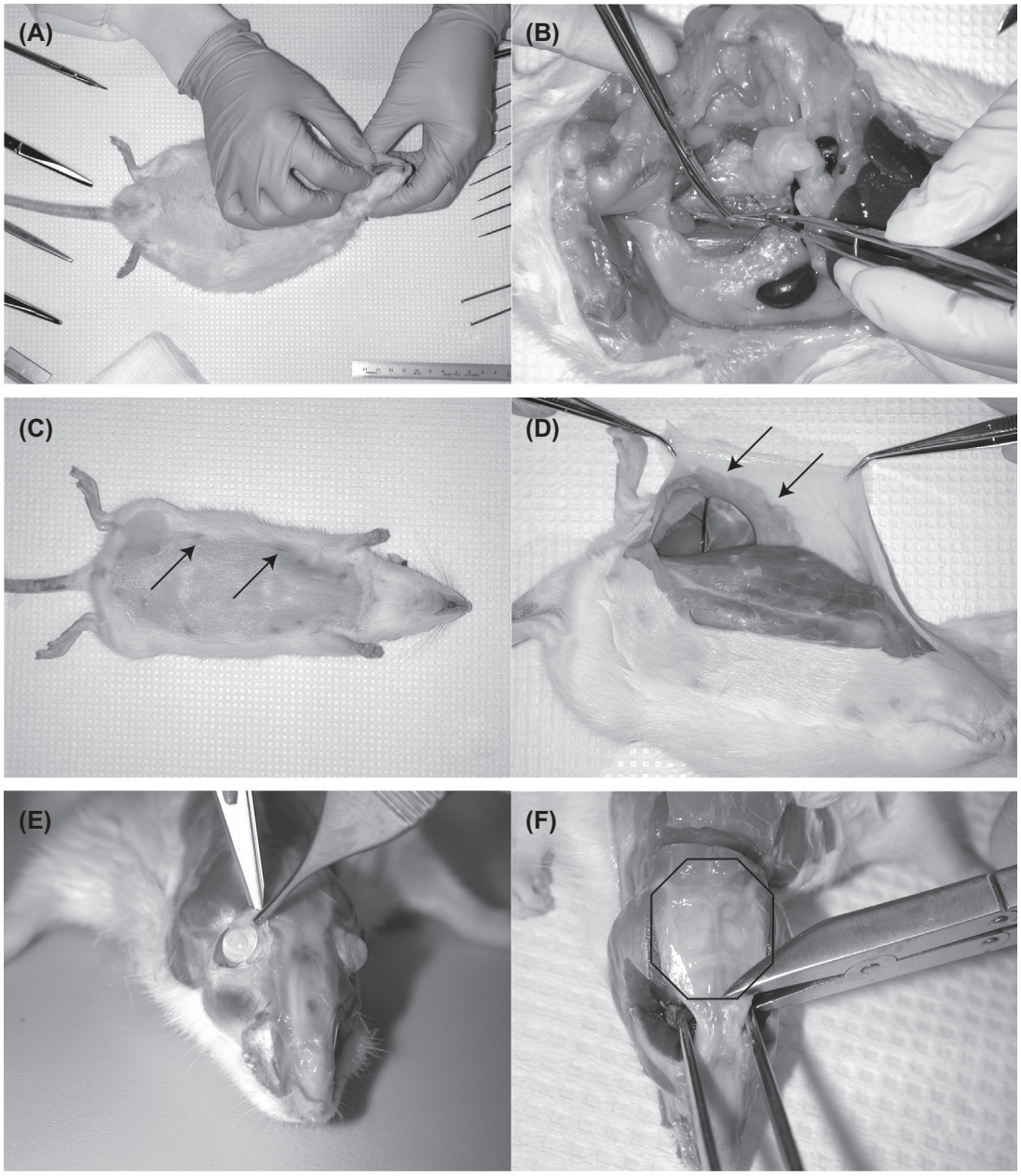

FIGURE 11.1 (A) Photograph of rat necropsy showing the prosector examining the oral cavity before initiating dissection. (B) Rapid exsanguination can be accomplished by severing the abdominal aorta. Note the location deep in the abdominal cavity. Overlying fat has been pushed aside. (C) Photograph of the ventral surface of a rat that has had the fur moistened to facilitate observation and dissection. *Arrows* show the location of mammary gland teats. (D) The *arrows* in this figure show the location of the mammary glands on the underside of the teats seen in panel c. (E) Eyes are removed with the optic nerve and Harderian gland attached by grasping the adnexa with forceps and cutting into the ocular orbit with a long narrow scalpel blade. (F) The brain is exteriorized by removing the calvarium. Shallow cuts are made with bone-cutting forceps along the lines indicated in this figure.

better histopathology or immunohistochemistry, and prevent pooled blood in tissues from obscuring lesions during the prosection process. After collection of blood samples according to protocol, rapid exsanguination of rats can be accomplished by severing the abdominal aorta (Fig. 11.1B), vena cava, or femoral arteries or by decapitation.

d. Dissection

The order of collection for tissues during any necropsy depends on the purpose of the study, on endpoints to be studied, and on which target tissues are of most importance. For the purposes of this chapter, a good general method for rat prosection with full screen tissue collection is presented.

Skin with mammary gland is collected first because it is easily forgotten when it is not a target tissue. If the protocol allows, the fur on the ventral surface of the rat can be moistened with saline or alcohol. This will prevent fur from getting into the other tissue samples. Two rows of teats can be located lateral to the midline from the cervical to the inguinal region (Fig. 11.1C). Grasping the fur, a section of skin that includes teat, skin, and mammary gland (Fig. 11.1D) is cut. This piece of tissue (fur side up) is then placed onto a piece of light cardboard or paper towel and into fixative. Note that when tissues are kept flat by placing them on a paper or cardboard substrate, there should be a short delay before they are carefully placed into the liquid fixative to allow fluid absorption and drying to create adhesion and prevent detachment.

If bone marrow is to be collected for impression smears, it is critically important to do so quickly because bone marrow cell morphology deteriorates rapidly postmortem.

The tissues from the head are dissected next because the brain autolyzes quickly after death. The eyes, with optic nerves attached and Harderian glands, are removed first by grasping the adnexa with forceps and cutting into the socket of the globe with a long narrow scalpel blade (Fig. 11.1E). Once the globe has been removed, the remaining fur is trimmed from the head, taking care not to damage the external nasal tissue. The nasal bones and frontal and parietal bones of the calvarium (skull) are easily identified. The brain is exteriorized by first removing the calvarium without disturbing the bones that cover the nasal cavity and turbinates. Shallow cuts made with bone-cutting forceps circumscribe the calvarium (Fig. 11.1F); it is then lifted off to expose the brain. Care is always taken to keep the cuts shallow enough to prevent damage to the brain tissue. The brain is removed with a small blunt spatula. The brainstem and optic nerves are cut so as not to stretch tissues, because stretching may cause artifact damage that will be noted by the pathologist during microscopic examination. Care is also taken to prevent damage to the pituitary gland that lies below the brain (Fig. 11.2A). This gland is generally removed after fixation.

The nasal cavity can be collected at this stage in the necropsy procedure or later with the remainder of the respiratory tract tissues. If it is collected at this stage, the lower jaw (with tongue) is separated from the head, and the head portion is cut from the carcass with scissors. The nasal cavity is flushed with fixative by inserting a blunt needle or cannula into the nasopharynx (Fig. 11.2B) and backflushing fixative slowly with a syringe until it exits the external nares.

At this stage in the necropsy, the abdominal and thoracic cavities (Fig. 11.2C and D) are opened with a ventral midline incision from the urethra to the jaw. The salivary glands and mandibular lymph nodes located in the cervical region are removed. The skin and musculature are reflected to expose the xyphoid cartilage. The thoracic cavity is opened by a cut through the diaphragm and ribs about 1 inch lateral to the midline. The lungs will collapse toward the spine and can be seen during removal of the sternum, ribs, and muscle. Blunt scissors are suggested for this portion of the dissection.

The tissues of the abdominal and thoracic cavities and the ventral cervical region are observed for macroscopic lesions. It is important to observe each organ in relation to neighboring organs, especially if lesions involve more than one tissue or displace organs. Masses that were palpated during the in-life stage or at the start of the necropsy are located, observed, removed, measured, described, and recorded at this time.

Those organs of the abdominal cavity that are easily lost should be located and dissected first. The mesenteric lymph nodes (Fig. 11.2E) are removed before the remainder of the gastrointestinal (GI) tract. The liver is removed by cutting the diaphragm and using a piece of this attached tissue to manipulate the organ. The liver hilus (where the lobes come together) can also be grasped with forceps to manipulate the organ. The spleen is removed next. The pancreas may be left attached to the spleen, attached to the stomach, or removed separately. Because of high enzymatic activity, care should be taken to keep the pancreas from contacting other tissues, and instruments used to dissect the pancreas should be rinsed off before contacting other tissues.

The entire GI tract (stomach to rectum) may be removed as a single structure (Fig. 11.2F), filled with fixative, and examined at a later time. Alternatively, each section of GI tract may be examined at necropsy. The stomach and cecum are opened along their greater curvatures to facilitate removal of ingesta and rinsing of the luminal surfaces with physiologic saline. The rat stomach has two regions: the nonglandular mucosa of the cardiac portion in the esophageal area, and the glandular epithelium of the pyloric part. The serosal and mucosal surfaces of the tubular GI organs are examined for lesions. These tissues may be placed on a card or paper to keep them flat during fixation. Adrenal glands are located cranial to the kidneys, embedded in perirenal fat (Fig. 11.3A). They are removed with some fat attached but later trimmed free after removal from the body. The kidneys with the ureters intact are examined for macroscopic lesions. If needed, kidneys can be individually identified as to left and right by a longitudinal nick in the greater curvature of the left kidney. The right kidney is cut in cross-section.

The thoracic viscera are removed in a single piece. This includes the tongue, larynx, trachea, thyroid gland,

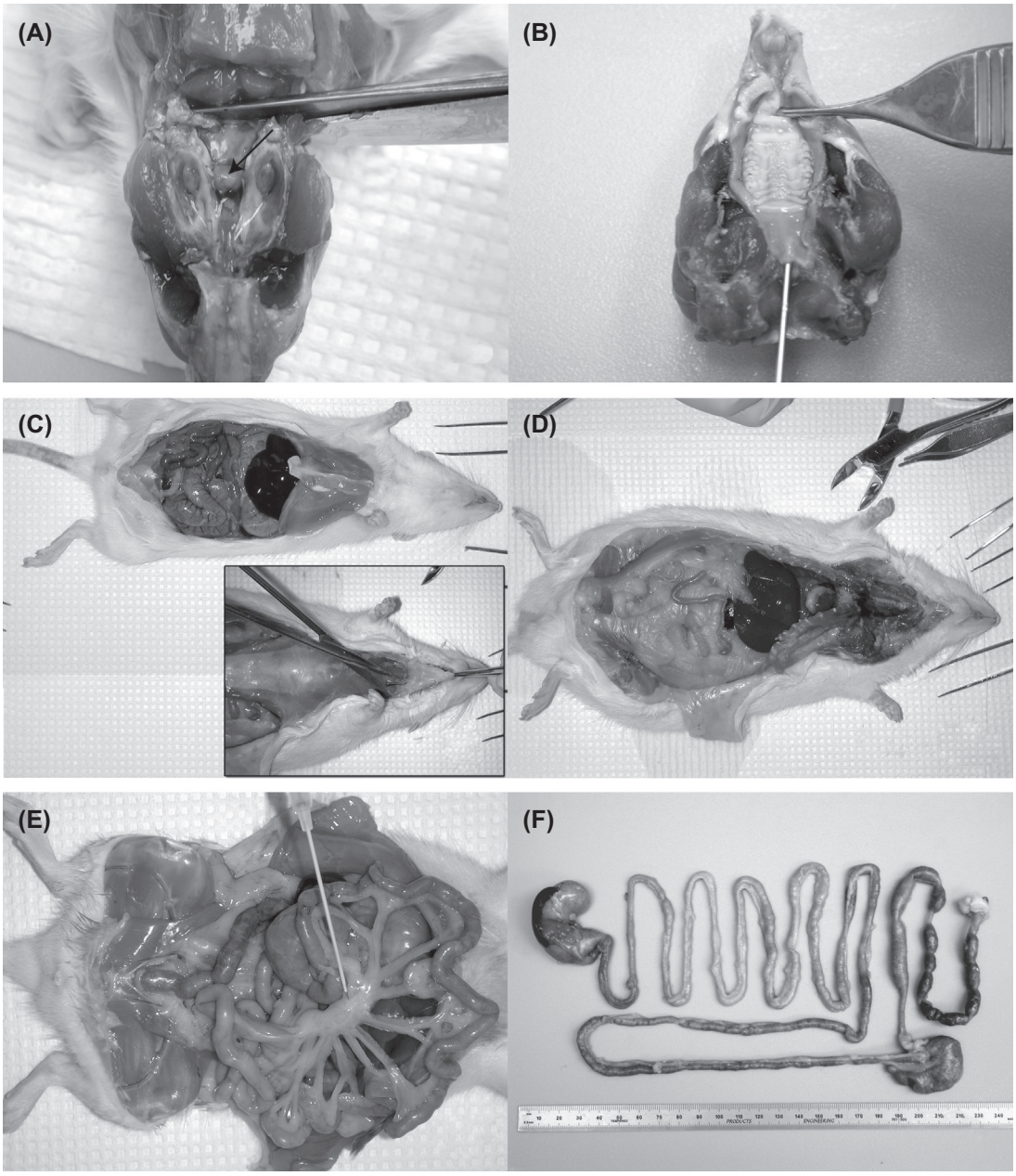

FIGURE 11.2 (A) After the brain has been removed, the pituitary gland can be visualized (*arrow*). This gland is generally removed after fixation. (B) The nasal cavity can be flushed with fixative by inserting a blunt needle or cannula into the nasopharynx and gently instilling fixative until it exits the external nares. (C) Photograph of rat necropsy with abdominal cavity opened to view the organs in situ. The *inset* shows the removal of the salivary glands and mandibular lymph nodes. (D) Photograph of rat necropsy after removal of the ribs and sternum to show the organs of the thoracic cavity in situ. (E) The mesenteric lymph nodes (located at the tip of the pointer) can be easily lost during the dissection of the abdominal organs. They are best located and removed before the other abdominal organs. (F) The entire gastrointestinal tract (stomach to the rectum) can be removed as a single structure.

parathyroid glands, esophagus, aorta, heart, thymus, lungs, and lung-associated lymph nodes. Lungs are often infused with fixative by syringe (to a fixed volume) or by pressure infusion (to a set pressure, usually 30 cm) either in the thoracic cavity (Fig. 11.3B) or after removal. The photomicrographs of Fig. 11.4 show rat lung tissue in both the desirable inflated and undesirable collapsed state. After infusion with fixative, the trachea is ligated to keep the tissue inflated until it has fixed.

Once the other abdominal viscera have been removed or placed aside, the urogenital track can be easily observed and dissected. Fig. 11.3C shows the male reproductive tract. To expose the testes in this manner, the epididymal fat is gently grasped so that the testes

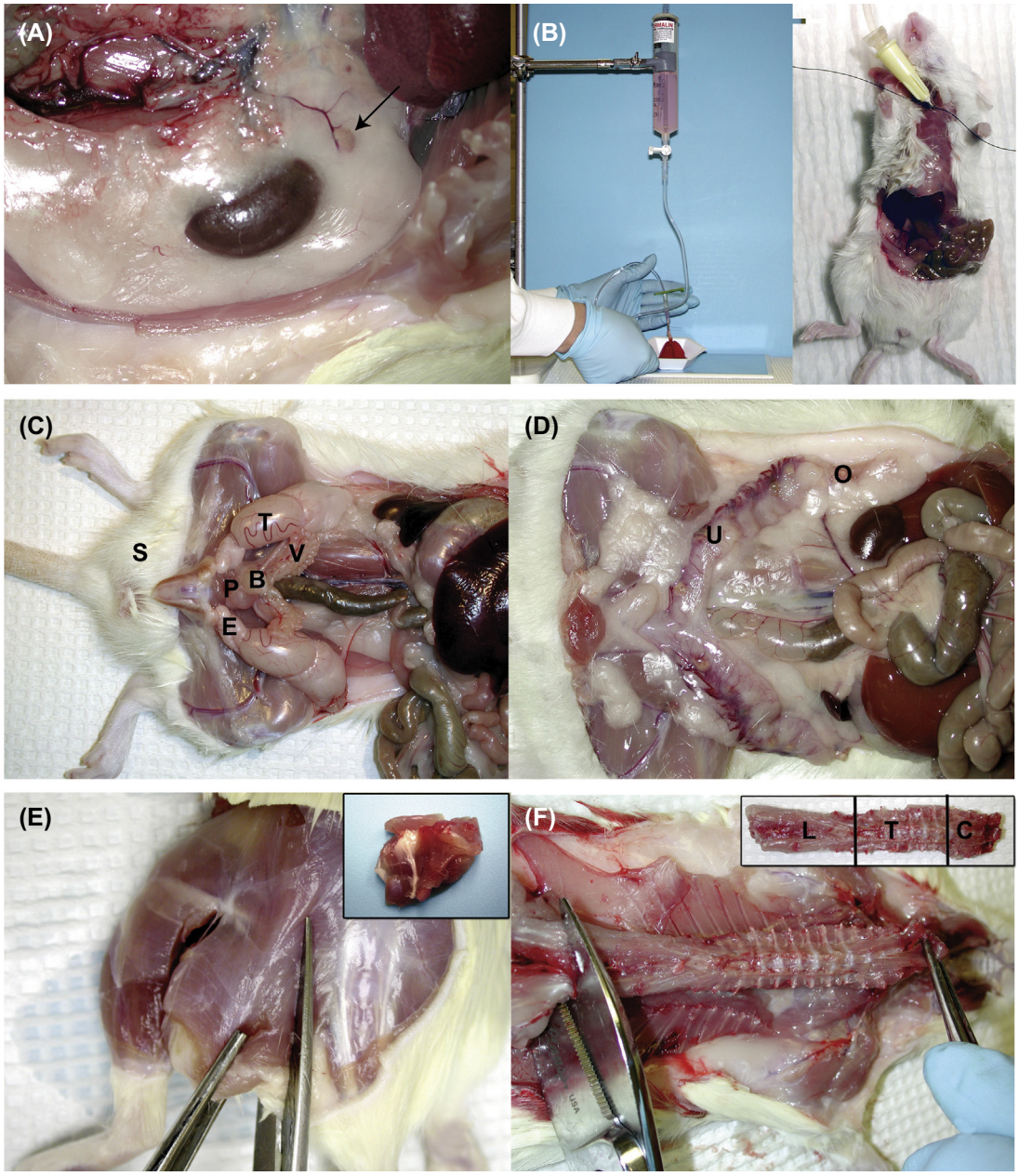

FIGURE 11.3 (A) Close-up of the rat abdominal cavity to demonstrate the location of the adrenal gland (*arrow*), embedded in fat and located cranial to the kidney. (B) Lungs may be infused with fixative to a set pressure (usually 30 cm) with a simple pressure infusion apparatus made from a syringe barrel, tubing, stopcock, and pipette. This can be performed after removal of the lungs from the animal or as the lungs are in the thoracic cavity. (C) Organs of the male reproductive tract. The testes (T) have been pulled from the scrotal sac (S); epididymis (E), seminal vesicles (V), urinary bladder (B), and prostate (P) are also visible. (D) Organs of the female reproductive tract. The uterus (U) and ovary (O) are visible. (E) This photograph shows the location for cuts made in the biceps femoris muscle, perpendicular to the femur for the collection of a standard skeletal muscle sample. After the sample is excised and flipped over, the sciatic nerve (*insert*) is visible. (F) Spinal cord may be collected from the cervical (C), thoracic (T), or lumbar (L) regions. Strong scissors or bone-cutting forceps are used to cut away the ribs and muscle.

slide easily from the scrotal sac. The epididymis may be left attached to the testis or carefully dissected free. Testis tissue is under pressure and any nick in the wall will cause seminiferous tubules to extrude from the testis, changing the orientation of tubules in the sample used for histopathology. Bone-cutting forceps or strong scissors are used to cut the pelvic girdle to remove the anus, penis, prostate, preputial glands, vas deferens, urinary bladder, and seminal vesicles in one piece. If the prostate is to be weighed, or certain lobes are to be weighed, it should be carefully trimmed and the lobes identified individually. Often the dorsal and lateral lobes

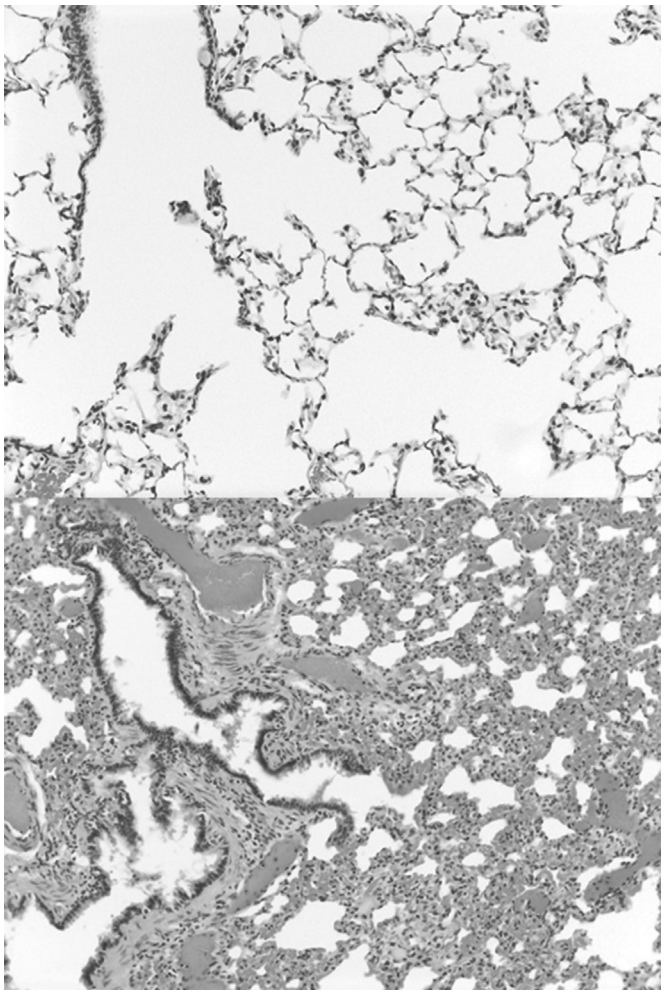

FIGURE 11.4 These photomicrographs show lung tissue that has been properly pressure infused with fixative (top) versus the appearance of lung tissue that was immersion fixed in a collapsed state (bottom). Inflation is necessary for proper microscopic evaluation of alveolar septa.

are combined and weighed separately from the ventral lobes (Suwa et al., 2001). In standard studies these organs can be fixed as a unit. If it is not already filled with urine, the bladder is filled with formalin by injection.

Fig. 11.3D shows the female urogenital system. Bone-cutting forceps or strong scissors are used to cut the pelvic girdle to remove the anus, rectum, vagina, urinary bladder, uterine horns, cervix, body of the uterus, and both ovaries. As with the male, the bladder may be inflated with fixative if it is not full of urine. The ovaries are dissected away from their surrounding fat and may be removed for weighing. As with the male, the reproductive viscera are generally fixed on paper or cardboard as a unit, but for studies with special emphasis on the female reproductive system, it may be preferable to dissect free some of these organs separately for weight determination and other endpoint analyses. If ovaries or other small tissues are dissected free, they may be placed in a tissue cassette to assure against loss.

Skeletal muscle is collected with sciatic nerve by skinning the dorsal surface of a hind limb. Two cuts are made into the muscle (biceps femoris) perpendicular to the femur about 1 inch apart, followed by one cut parallel to the femur, close to the bone (Fig. 11.3E). The piece of muscle is excised and flipped over to expose the sciatic nerve (Fig. 11.3E, insert). The remaining hindlimb may be saved for bone marrow or knee joint collection.

Spinal cord for routine studies is collected from the thoracic region (Fig. 11.3F). Bone-cutting forceps or strong scissors are used to cut away the ribs and muscle. At least two cuts are made into the vertebrae of each region to allow fixative to penetrate. If lumbar spinal cord is to be collected, prosectors should collect in the L1−L2 vertebral region to ensure a cord and not spinal nerve root sample. Some protocols in use call for the collection of a representative spinal cord section from the cervical, thoracic, lumbar, and sacral regions.

Pathology Considerations: It is critically important for the prosector to observe, describe, and record all macroscopic abnormalities that might influence the interpretation of subsequent studies such as histopathology (Ward and Reznik, 1983). To accomplish this task, it is important to understand pathology terminology and develop a standardized lexicon for use. Terms associated with medical and pathological diagnoses are complex and specialized. Terms associated with gross necropsy description are best kept simple and descriptive. There is no one standard set of terms or way to describe findings. Good working examples of systems have been reviewed (Feldman and Seely, 1988; Bono et al., 2000). Because a pathology diagnosis will be made from the collective microscopic and clinical data, a good description of the location, size, color, shape, texture, and severity of any abnormality seen is adequate to assure further examination. Some terms and examples are presented in Table 11.5.

3. *Tissue Preparation*

Fixation of tissues is one of the basic processing steps used in histology. Fixatives are chemicals or physical processes that are used to "fix" in time the relationships among cells, cellular components, and extracellular matrix (Jones, 2001). Unfortunately, each combination of fixation, processing, and staining is a compromise as to the best representation of the living tissue. Thus there is no ideal fixation protocol, and one must bear in mind the purpose and needs of the study and the endpoints being studied. Chemical fixation is a complex topic for which there are many excellent reviews (Eltoum et al., 2001; Jones, 2001). The advent of molecular pathology has increased the need for a prospective

TABLE 11.5 Common Criteria and Terms Used to Characterize Necropsy Findings.

Morphology	Adhesion
	Reduction in size
	Discoloration
	Focus
	Mass
Location	The organ, limb, or cavity where the lesion is located, with directional or organ-specific terms
Size	Use two or three dimensions (largest first)
Weight	In grams, for organs and lesions; record significant figures with consistency
Color	Use standard real colors (no "-ish") and patterns
Shape	Examples include nodular, spherical, ovoid, crateriform, polypoid
Texture (consistency)	Examples include soft, firm, hard, gritty
Severity	Not graded (0)
	Minimal (1)
	Mild (2)
	Moderate (3)
	Severe (4)

consideration of fixation protocols. One should always avoid "overfixation" in aldehyde-based fixatives such as the 10% neutral buffered formalin used as the most common fixative in the laboratory. Overfixation can impair certain histological analyses such as immunohistochemistry. For routine histology, tissue specimens are fixed by immersion in 10% neutral buffered formalin solution for 24–48 h. Many research necropsy procedures, such as those that involve nervous tissue examination or special target organ studies, will involve intravascular perfusions of fixative using flow and pressure control. The best way to avoid overfixation is to fix for a specified amount of time and then transfer tissues to 70% alcohol or buffer or to process quickly to paraffin.

For immersion fixation techniques, commonly used rules of thumb suggest that the volume of fixative should always exceed that of tissue specimens by at least 10–20-fold to maintain adequate fixative concentrations throughout the process (Knoblaugh et al., 2012). For rapid and uniform fixation, tissues should not be more than 3–5 mm thick. Other commonly used fixatives for rats include Bouin's solution for reproductive and fetal tissues, Davidson's fixative for ocular and male reproductive tissues, and Zenker's solution for eyes. For ultrastructural studies using electron microscopy, glutaraldehyde and Karnovsky's fixative are commonly used on very small pieces of tissue. Many of these fixatives entail hazardous agent handling and special waste-handling considerations. For example, formalin is a carcinogenic substance, Bouin's solution has picric acid that can be explosive under certain conditions, and Zenker's solution contains mercury, a toxic material that can be readily absorbed into the body.

Modern pathology combines traditional histology with molecular biology techniques and requires the scientist to give special consideration to the collection of tissues and specimens for techniques such as immunohistochemistry, in situ hybridization, and in situ PCR (Eltoum et al., 2001). This may necessitate specific tissue collection protocols that preserve biological targets in special ways. Rapid freezing of specimens with or without cryoprotectants and/or special fixatives are some examples. RNA-based techniques may require special handling to prevent enzymatic destruction of targets. These include the use of RNAase-destroying solutions and the protection of all surfaces that contact tissues, such as the use of gloves and disposable cryostat blades. As the use of genetically modified rats grows, the need for collection of tissues for molecular analysis will grow.

4. *Interfacing the Rat Necropsy With the Microscopic Evaluation of Tissues*

After fixation, tissues need to be trimmed and then processed for embedment into paraffin or plastic media for subsequent microtomy and histologic preparation. The tissue trim procedure for rat tissues requires standardization so that the pathologist is presented with adequate specimens to evaluate. Good tissue trim involves uniform sample size, sampling from standard regions, maintaining natural borders, maintenance of orientation, and maintenance of tissue identification. Tissue trim methods for the rat have been described (Bahnemann et al., 1995; Bono et al., 2000). After tissue trim, rat tissues are generally multiple embedded. A complete full screen tissue examination from a rat will generally involve approximately 60 tissues distributed on 18–20 hematoxylin/eosin-stained slides, but for routine necropsy cases there may be a more limited targeted tissue list and a corresponding smaller number of slides.

5. *Screening of Tissue by Histology for Lesions of Infectious Disease*

A variety of tissues may be screened for lesions indicative of infectious disease. While there are very few pathognomonic lesions of rodent infections, screening

of tissues may provide presumptive diagnoses that can be confirmed by other means. The disadvantages of histology as a screening tool include the narrow window of opportunity to detect certain transient infections and the fact that many opportunistic pathogens do not cause histologic disease. Screening of tissues may be useful in several situations: (1) screening of target tissues for known lesions of infectious disease; (2) screening immunodeficient rats in which tests such as serology are not appropriate; (3) detecting disease early in its time course prior to the development of detectable antibody; (4) detecting bacterial agents that are difficult to cultivate (such as *F. rodentium* and *Clostridium piliforme*); and (5) detecting emerging or previously unrecognized infectious diseases. The latter is exemplified by the recent discovery of RatPyV2 (Besch-Williford et al., 2017). This agent was discovered by histological examination of multiple tissues demonstrating multifocal intranuclear inclusions located within epithelial cells of the respiratory tract, salivary glands, and lacrimal glands (Besch-Williford et al., 2017; Rigatti et al., 2016). In addition, it is only by histopathology that noninfectious degenerative conditions, such as renal and cardiac calcinosis, may be recognized.

The use of tissue screening for infectious disease relies upon the selection of certain target tissues. It is unrealistic to screen all tissues for signs of disease and many tissues are not common sites of infection. Most commonly, systems exposed to the external environment (for example, respiratory and enteric systems) are screened. Other tissues often screened are based on known disease pathogeneses. These include the Harderian and salivary glands, which are screened for lesions of RCV/SDAV, and the urinary bladder, which is screened for *Trichosomoides crassicauda* infections.

a. Serology

Examination for antibodies produced during an infection is one of the most economical and efficient means of screening rats for infectious disease. Sample types suitable for serological testing include serum, plasma, and dried blood spot (DBS) specimens. DBS sampling involves the collection of a single drop (~25 μL) of whole blood onto filter paper. The DBS sample, once dry, is very stable and amenable to a variety of quantitative and qualitative analyses. DBS sampling was first introduced in 1963 as a means to measure phenylalanine levels for the diagnosis of phenylketonuria in newborns (Guthrie and Susi, 1963). Since that time, DBS samples have been implemented in newborn metabolic disease screening, molecular testing for infectious disease diagnosis, therapeutic drug monitoring, and preclinical pharmacokinetic and toxicokinetic testing in rodents. Serology offers several advantages: (1) samples can be obtained from a euthanized, anesthetized, or restrained rat; (2) multiple tests can be performed on a single sample; (3) antibodies (IgM followed by IgG) are detectable 1−2 weeks following exposure to the infectious agent; (4) serum antibody is long lasting (months), so the organism does not need to persist in the host for the infection to be detected; and (5) the antigens used in serologic assays can be highly purified, rendering these tests very sensitive and specific (Hsu et al., 2007; Livingston and Riley, 2003).

A variety of serologic methodologies have been developed, including the enzyme-linked immunosorbent assay (ELISA), the immunofluorescence assay (IFA), and more recently the multiplex fluorescent immunoassay (MFI). The Western blot assay is a valuable adjunct test for MFI, ELISA, and IFA but is not commonly used as a primary test. Other methods such as hemagglutination inhibition, complement fixation, and serum neutralization are time consuming and not as sensitive as MFI, ELISA, or IFA and are thus no longer routinely used in rat infectious disease diagnosis.

ELISA has long been the gold-standard immunoassay for detection of pathogen-specific antibodies in laboratory animals. It is classically constructed by coating a 96-well microtiter plate with the antigen of interest and detection of the analyte is achieved through enzyme-mediated amplification of the signal. In contrast, MFI utilizes a microsphere array as the solid phase for antigen coating and detection of analyte is accomplished with a fluorescent reporter. MFI offers several advantages over traditional ELISA, including: (1) simultaneous detection of antibodies to as many as 100 different antigens in a single reaction well; (2) as little as 1 μL of undiluted serum is required per reaction well, regardless of the number of different microsphere−antigen complexes in the reaction well; (3) multiple antigens for a single agent can be evaluated simultaneously, offering internal confirmation of exposure to that agent if antibodies are detected in the sample; (4) compatibility with various sample types (serum, plasma, and DBS); and (5) the fluorescence reporter provides a greater dynamic range of detection and generally greater sensitivity (Hsu et al., 2007; Khan et al., 2005; Ravindran et al., 2010; Schmidt et al., 2017). MFI is also adaptable to automation; thus a large number of samples can be rapidly screened. The workflows for ELISA and MFI are similar. In general, indirect serological tests utilize antigen bound to a solid phase (ELISA, 96 well plates; MFI, microspheres). The specimen is then incubated with the solid phase and if antibody to the antigen is present, it will bind in a specific manner. Antibodies not specific for the antigen are removed in subsequent washing steps. Following the wash steps, conjugated (ELISA, enzyme; MFI, biotin) antirat immunoglobulin secondary antibody is added. These bind to rat antibodies that were bound in the first

step. The last step involves the addition of a reporter (ELISA, enzyme substrate; MFI, streptavidin–phycoerythrin). The intensity of the reporter signal (ELISA, colorimetric; MFI, fluorometric) is proportional to the concentration of agent-specific antibody present in the specimen.

IFA methodology is similar in principle to ELISA and MFI (Kendall et al., 1999). Briefly, bacteria or virus-infected cells affixed to wells of a glass slide are probed with a test specimen. Following a wash step, fluorescent dye-conjugated antirat immunoglobulin secondary antibody is added, washed, and slides are read with an epifluorescence microscope. The added advantage of the IFA is that the cellular location and pattern of fluorescence can be evaluated to differentiate specific and nonspecific reactivity. For example, predominant cytoplasmic or nuclear fluorescence may be consistent with certain viral infections as opposed to diffuse fluorescence, which may indicate a nonspecific reaction. IFAs are relatively inexpensive, but more expensive than ELISA and MFI. The major disadvantages of IFA are that it is labor intensive and interpretation is dependent on the expertise of the diagnostician. The choice between ELISA, MFI, and IFA is based on personal preference of the laboratory. These tests are often used in combination, with the ELISA and/or MFI serving as the primary test modality and the IFA serving as a confirmatory test.

As described earlier, understanding the ecology of infectious disease in laboratory animals is imperative to the success of biomedical research and relies heavily on robust health monitoring programs. Serological surveillance has provided a bulwark against the spread of infectious agents for several decades and has performed a central role in improving the health standard for rodent research models. The major advantage of serology is that agent-specific antibodies persist for months to years despite the transience of the agent. This footprint of infection provides a large window of opportunity to diagnose infections; however, serologic testing at a single time point cannot distinguish active from prior infections. Although serology has few limitations, it is unreliable in the diagnosis of infections in immunodeficient rodents (Compton and Riley, 2001; Livingston and Riley, 2003). MFI, ELISA, and IFA are subject to nonspecific reactivity, which can lead to false-positive results. This is especially true in bacterial serological assays due to the complexity and abundance of potentially cross-reactive bacterial antigens. With improvements in antigen production and reagents, false-positive results are uncommon. However, because of this possibility, a single positive should always be confirmed with additional testing. Nonspecific reactivity may also occur in serum from aged rodents over 6 months old (Wagner et al., 1991), strains subject to autoimmunity, animals whose immune systems are nonspecifically stimulated because of injury, neoplasm, other noninfectious disease processes, or other types of antigenic stimulation (Wagner et al., 1991; Weisbroth et al., 1998).

Antigens employed in serology testing vary in complexity from crude extracts containing multiple antigens and impurities to select recombinant proteins generated in viral vectors. The use of highly purified subunit antigens may increase specificity as cross-reactive impurities are not present. However, the use of these subunit antigens may negatively impact sensitivity (Compton and Riley, 2001). This happens because the host response is polyclonal, with many antibodies being produced to different antigenic epitopes on the infectious agent. Highly purified subunit antigens may lack the immunodominant epitopes to which antibodies have been produced and result in a test with decreased sensitivity when compared to one that utilizes crude protein preparations. Moreover, agents may express different epitopes during different stages of disease. Therefore an assay that uses an antigen that is only expressed at certain stages may miss some infections. In practice, a balance is sought so that purified preparations of multiple antigens are used, resulting in very sensitive and specific assays.

b. Culture

Culture of bacteria may be incorporated into health-monitoring programs. Culture is especially useful when clinical evidence of bacterial infection is present (e.g., peritonitis, pyometra, and abscessation). Culture is most effective during the height of infection, and prior to administration of antibiotics or the development of an immune response (Compton and Riley, 2001). Culture may also be used as a screening tool for pathogens or agents capable of causing opportunistic infections. In the latter scenario, mucosal sites of the intestinal tract (for example, the cecum) and respiratory tract (for example, the nasopharynx) are cultured on broad spectrum or selective media. Culture has the advantage of determining whether a live agent is present, as opposed to potentially nonviable DNA remnants or antibacterial antibodies from a past infection. Culture does have some drawbacks. For example, agents colonizing the mucosal surface may be present in low numbers or sequestered in areas not accessed by routine procedures (for example, the deep recesses of the nasal turbinates). Moreover, fastidious organisms may not grow well unless conditions are optimized, or their growth may be hindered by the growth of more vigorous bacteria. Some agents may take several days to grow into identifiable colonies, while some agents such as *F. rodentium* and *C. piliforme* have yet to be cultivated on cell-free media. Collection of samples for submission to diagnostic laboratories may also be

problematic in that some bacteria, notably the Pasteurellaceae, do not survive well in transfer media.

Traditionally, bacterial speciation has been based on colony morphology, Gram-staining characteristics, organism morphology, biochemical tests, and growth on selective media or in selective conditions (Feldman and Feldman, 2001; Livingston and Riley, 2003). More recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has emerged as a revolutionary tool for microbial identification (Singhal et al., 2015; Goto et al., 2012). MALDI-TOF MS is based on laser-induced ionization and desorption of microbial proteins and peptides. The protonated ions are accelerated in a vacuum containing a fixed high-voltage electric field. The time required for the microbial proteins to reach the detector is a direct function of the mass to charge (m/z) ratio, creating a peptide mass fingerprint (PMF). The PMF pattern, representative of highly abundant ribosomal proteins, is unique for each species and is used to speciate microorganisms by matching the PMF pattern of an unknown specimen with the PMF patterns of highly characterized microorganisms contained in the database. Fig. 11.5 shows the PMF of *Staphylococcus aureus*. MALDI-TOF MS improves the precision and rapidity of microbial identification compared to traditional methodologies. Culture and subsequent biochemical or MALDI-TOF MS analyses are very specific for most agents and can be supplemented with molecular techniques where precise speciation or strain identification is desired.

c. Molecular Diagnostics

Molecular diagnostic techniques, primarily those based on the PCR technique, are rapidly replacing traditional diagnostic methodologies (Compton and Riley, 2001). PCR utilizes specific oligonucleotide primers to exponentially amplify small amounts of target DNA or RNA from a particular organism that is present in a clinical specimen. PCR offers exquisite sensitivity and specificity, detecting as few as 1−10 template copies (Compton and Riley, 2001), and is readily adapted to the detection of bacterial, viral, parasite, and fungal agents.

Details of the PCR technique can be found in a number of technique manuals. Briefly, PCR consists of repetitive cycles of a three-step amplification procedure. Double-stranded sample DNA is denatured into two single strands. Oligonucleotide primers specific for the agent (complementary to the specific microorganism genome and typically situated approximately 100 base pairs apart) are added and allowed to anneal to target sequences in the sample DNA. A polymerase (for example, Taq polymerase), an enzyme that functions in DNA synthesis, is added along with nucleotide bases and new DNA strands of a specific size are created. After "n" cycles of this three-step process of denaturation, annealing, and synthesis, the target sequence is amplified 2^nth times (30 cycles $= 2^{30} = 1{,}073{,}741{,}824$ copies of DNA). RNA (RNA viral genomes) may also be detected by PCR. In this case, reverse transcriptase PCR (RT-PCR) is utilized. With RT-PCR, RNA is converted to complementary DNA (cDNA) using the enzyme, reverse transcriptase. The cDNA then becomes the template for PCR. The PCR product may then be subjected to gel electrophoresis, and if a targeted sequence is amplified, it will migrate to a specific location in the gel based on its molecular weight. Real-time PCR assays using fluorescently labeled sequence-specific oligonucleotide probes are commonly used for rodent health monitoring (Feldman and Feldman, 2001; Besselsen et al., 2002, 2003; Drazenovich et al., 2002; Uchiyama and Besselsen, 2003). These assays offer improved sensitivity and specificity, require no post-PCR processing, and can be used to quantify infectious agents.

PCR offers superior sensitivity and specificity, and results can be obtained in a single working day. The main disadvantages of PCR are directly related to its advantages. The exquisite sensitivity renders contamination especially problematic, and false positives may occur if strict laboratory technique protocols are not in place or followed. The test is also relatively expensive due to

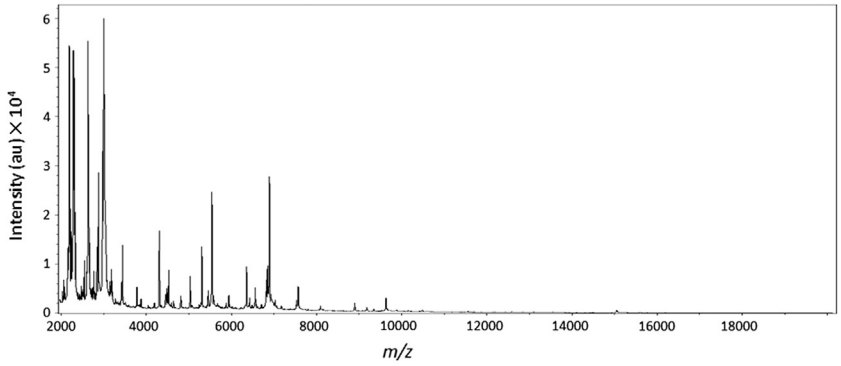

FIGURE 11.5 Peptide mass fingerprint (PMF) profile of *Staphylococcus aureus*. PMF was constructed as a mean value of 240 mass spectra using MALDI Biotyper 3.0 software. The m/z ratio is shown on the x-axis and the intensity values is shown on the y-axis.

the need for expensive equipment and its labor intensiveness. This expense can be partially overcome by pooling of samples. In addition, costs will likely be lowered as technology allows for more automation of PCR. The expense of PCR also relates to the need for multiple samples from which multiple tests must be performed. Lastly, PCR is often performed on biological samples, many of which contain inhibitors of components of the PCR reaction such as heme and plant products that contaminate feces (Panaccio and Lew, 1991; Al-Soud and Radstrom, 2001; Compton and Riley, 2001; Feldman and Feldman, 2001). This possibility must be considered when testing these samples; however, the use of highly purified DNA can eliminate or sufficiently dilute inhibitors, so that accurate results are obtained.

Sampling for PCR requires knowledge of the pathogenesis of the agent, including tissue tropism and duration of infection (Compton and Riley, 2001). PCR is an ideal primary test for the detection of active or persistent infections (for example, infections by parvoviruses, lymphocytic choriomeningitis virus (LCMV), *Mycoplasma pulmonis*, *Helicobacter* spp.) or those agents for which other diagnostic tests are of poor sensitivity (for example, the culture of *Helicobacter* spp.). In contrast, detection of infections where colonization is transient (many viral infections) is possible for only brief periods of time. In the latter case, PCR may serve as an adjunct test. In this scenario, infections may be detected by a primary test such as serology. To confirm infection, additional rats of appropriate target age (an age at which colonization or shedding is expected) are selected and target specimens are tested by PCR. This two-methodology approach provides very convincing evidence of infection. Moreover, although PCR cannot distinguish between live and dead organisms, results from PCR testing can provide valuable information about the current status (actively shedding, free of colonization) among individual animals or groups of animals. The use of exhaust air debris environmental samples as an alternative/adjunct to sentinel and direct experimental animal sampling has been studied primarily for health monitoring of mice. These studies demonstrated that PCR testing of exhaust air debris environmental samples from racks with unfiltered exhaust air flow reliably detected the presence of fur mites, pinworms, *Helicobacter* spp., *R. pneumotropicus*, and protozoa (Bauer et al., 2016; Brielmeier et al., 2006; Jensen et al., 2013; Kapoor et al., 2017; Manuel et al., 2017; Miller et al., 2016; Zorn et al., 2017; Compton et al., 2004). While it is tempting to extrapolate these findings, further studies are needed to evaluate the impact of variables such as rack type, air flow, bedding type, and prevalence of infection on sample reliability specifically for rats.

Other molecular methodologies such as next-generation sequencing, which allows for the simultaneous screening of hundreds of agents, will surely add to the arsenal of molecular techniques available to the diagnostician in the near future (Chiu, 2013; Deurenberg et al., 2017; Forbes et al., 2017; Kim et al., 2018; Simner et al., 2018). Molecular techniques are also applicable in many other areas of rat medicine and biology, including the detection of contaminants in tissue culture material and monitoring of genetic purity of inbred strains or genetically engineered rats (Bryda and Riley, 2008; Shimoyama et al., 2017).

d. Parasite Screening

Screening for parasites is usually accomplished by a subgross or microscopic examination of parasite niches. The three general classes of parasites that infect rats include ectoparasites (mites and lice), endoparasitic helminths (pinworms, other nematodes and cestodes), and endoparasitic protozoa. For ectoparasites, the pelage can be collected and examined for mite or louse infestation. Most protocols suggest allowing the sample to cool to encourage mites to venture to the tips of the hair shaft in search of a warmer host. Alternatively, Scotch tape tests may be effective for detecting mites or mite eggs attached to hair shafts. The latter can also be utilized in the live animal.

Endoparasitic helminths may be detected by direct examination of the intestinal tract for adult worms. *Syphacia muris* pinworms usually inhabit the cecum and *Rodentolepis* (*Hymenolepis*) spp. tapeworms the small intestine. Detection of helminths in gross specimens may be enhanced by the use of a dissecting microscope. Incubation of a section of intestine in saline for a short period of time may also facilitate detection by allowing worms to migrate out of the dark fecal matter into the more transparent saline. Pinworms of the genera *S. muris* also deposit ova on the perineum and can thus be detected by perineal tape testing. For this test, a piece of clear cellophane tape is applied to the perineal skin, placed on a microscope slide, and examined for typical banana-shaped ova. This test offers the advantage of being usable in live animals. As an alternative, fecal flotation may be used to detect pinworm or cestode ova.

Endoparasitic protozoa are usually commensal organisms of questionable pathogenicity. These agents are generally detected by wet mount preparations of intestinal contents. Protozoa are readily identified based on motility, morphology, and intestinal locale. For example, *Spironucleus muris* is most often found in the small intestine and is characterized by its small teardrop shape with darting motility. *Giardia* sp. are also found in the small intestine but are larger, have a cup-shaped morphology with an "owl face" appearance, and a "falling leaf" motility. Other protozoa include trichomonads (lemon shaped with undulating membrane and rolling motility), *Chilomastix* sp. (oval to bar shaped with

spiraling motility), and *Entamoeba* sp. (ameboid shaped with slow motility by pseudopod formation).

In general, patent infections by parasites are more readily detectable in young animals (Wagner et al., 1991; Weisbroth et al., 1998). Microscopic and gross examinations for parasites are advantageous in that they are relatively simple, straightforward techniques; some (tape tests, fecal flotation) can be performed on live animals and they are relatively specific. The disadvantage of these tests is that they lack sensitivity for detection of light to moderate infection and are thus quickly being replaced by more sensitive PCR (Jackson et al., 2013; Gerwin et al., 2017). Indeed, some contemporary health-monitoring programs routinely employ PCR for parasite screening. Infectious agents that are not efficiently transmitted to sentinel rats via dirty bedding (e.g., endoparasites and fur mites) are more reliably detected in samples taken directly from colony rats. Optimal sample types include feces for the detection of endoparasites (e.g., *Entamoeba* sp., *Giardia* sp., pinworms, *Rodentolepis* sp.) and fur swabs or cage swabs for the detection of mites (e.g., *Ornithonyssus bacoti* and *Radfordia ensifera*). PCR testing of large numbers of samples is costly. To offset the cost, individual samples are routinely pooled. PCR offers exquisite sensitivity, detecting as few as 1−10 template copies (Compton and Riley, 2001); therefore individual samples may be pooled in groups of $\leq$10 with little concern for loss of diagnostic sensitivity.

e. Other Testing Strategies

Historically, other testing platforms were employed, including stress testing for *C. piliforme* (Fries and Ladefoged, 1979) or *P. carinii* (Armstrong et al., 1991). These tests may still be used as a diagnostic tool or for the characterization of a novel pathogen, but they are rarely if ever used in routine health monitoring. Moreover, certain strains of rats, such as gnotobiotic or axenic rats, may require additional tests, such as microbiome analysis, that can be coupled with health monitoring.

Table 11.6, adapted from Livingston and Riley (Livingston and Riley, 2003), lists agents commonly tested for in rat health monitoring programs and methodologies used to test these agents.

C. Testing Profiles

Tests used in the monitoring of rats for infectious disease are often packaged, depending on the institution's needs, into profiles that include one or more testing modalities. These profiles almost invariably include serologic examination for antibodies to viral and bacterial agents and may include gross necropsy examination, parasite examination, examination for enteric or respiratory pathogens using culture or molecular techniques, and histologic examination of target tissues. The design of these testing profiles requires consideration of several factors as outlined in earlier sections of this chapter. Tiered testing strategies are very economical and are becoming commonplace (Laber-Laird and Proctor, 1993). With these strategies, the most prevalent agents are tested for on a frequent basis via inexpensive high-throughput tests, while testing for agents of low prevalence or screening of animals for indication of emerging diseases occurs on a less frequent basis. For example, rats may be screened for endoparasites, ectoparasites, *C. piliforme*, *Helicobacter* spp., *M. pulmonis*, *R. pneumotropicus*, *R. heylii*, *R. ratti*, parvoviruses, PVM, RTV/Boone cardiovirus, and RCV/SDAV on a quarterly basis, whereas less prevalent agents such as *Corynebacterium kutscheri*, *F. rodentium*, *S. moniliformis,* adenovirus, LCMV, reovirus, Sendai virus, and Seoul virus are tested for once a year (Nicklas et al., 2002; Mahler et al., 2014; Pritchett-Corning et al., 2009).

D. Test Interpretation and Retesting

In many cases, interpretation of health monitoring results is straightforward. For example, when several rats with cervical swellings are found to be seropositive for RCV/SDAV, it is reasonable to determine that an outbreak of this infection is occurring. In other cases, the diagnosis is not so clear-cut, and test results require careful interpretation and follow-up testing. For example, in cases where a single animal is found to be seropositive for PVM, this may indicate either an early outbreak or a false-positive result.

There are several approaches to test interpretation. First, results should be interpreted in the context of the entire colony and the health monitoring program. Decisions about rodent health should rarely if ever be made on a single positive result and the latter should be assumed to be a false positive until verified (Laber-Laird and Proctor, 1993; Compton and Riley, 2001; Livingston and Riley, 2003). Verification may include testing a sample (serum) using an alternative test platform, testing a second sample from the affected animals using an alternative test platform (for example, through PCR), or testing cohort animals (Livingston and Riley, 2003; Weisbroth et al., 1998). As discussed earlier, there are three primary serologic testing platforms: ELISA, MFI, and IFA. These platforms can also be used as adjuncts for each other. In most diagnostic laboratories, the MFI and/or ELISA serves as the primary serological test, and borderline or solitary positive results are confirmed by IFA. Confirmatory testing may also involve the use of different testing platforms. For example, if a rat is found to be seropositive for *F. rodentium*, the lungs may be tested by PCR or examined with a silver stain to detect

TABLE 11.6 Commonly Used Testing Methodologies for Rat Pathogens.

Agent (Species)	Primary Testing Methodology (Sample Tested)	Confirmatory Testing Methodology
VIRUSES		
Hantavirus (Seoul)	Serology (DBS, serum)	PCR (feces, kidney)
Lymphocytic choriomeningitis virus (LCMV)	Serology (DBS, serum)	PCR (feces, kidney)
Mouse adenovirus type 1 (MAV1)	Serology (DBS, serum)	PCR (feces, lung)
Mouse adenovirus type 2 (MAV2)	Serology (DBS, serum)	PCR (feces, intestine)
Pneumonia virus of mice (PVM)	Serology (DBS, serum)	PCR (feces, trachea, lung)
Rat coronavirus (RCV/SDAV)	Serology (DBS, serum)	PCR (feces, salivary and Harderian glands)
Rat parvoviruses	Serology (DBS, serum)	PCR (feces, mesenteric lymph node)
Rat polyomavirus 2 (RatPyV2)[a]	Serology (DBS, serum)	PCR (feces), histology (salivary gland, trachea)
Rat theilovirus (RTV)	Serology (DBS, serum)	PCR (feces, intestine)
Boone cardiovirus[b]	Serology (DBS, serum)	PCR (feces)
Reovirus type 3 (REO 3)	Serology (DBS, serum)	PCR (feces, intestine)
Sendai virus (Sendai)	Serology (DBS, serum)	PCR (feces, trachea, lung)
BACTERIA		
Filobacterium rodentium (CAR bacillus)	Serology (DBS, serum)	PCR (feces, trachea), histology (trachea, lung)
Clostridium piliforme	Serology (DBS, serum)	PCR (feces), histology (feces, intestine, liver)
Corynebacterium kutscheri	PCR (feces, oral swab)	Culture (nasopharynx, oral swab)
Helicobacter spp.	PCR (feces)	Culture (feces, cecal contents)
Mycoplasma pulmonis	Serology (DBS, serum)/PCR (feces, oral swab)	Culture (nasopharynx)
Rodentibacter pneumotropicus[c]	PCR (feces, oral swab)	Culture (nasopharynx, oral swab)
Rodentibacter heylii[c]	PCR (feces, oral swab)	Culture (nasopharynx, oral swab)
Rodentibacter ratti[c]	PCR (feces, oral swab)	Culture (nasopharynx, oral swab)
Salmonella spp.	Culture (cecal contents, feces)	PCR (cecal contents, feces)
Streptobacillus moniliformis	PCR (feces, oral swab)	
Streptococcus pneumoniae	PCR (feces, oral swab)	Culture (nasopharynx, oral swab)
PARASITES		
Ornithonyssus bacoti	PCR (fur/cage swab), Direct exam (pelage)	
Radfordia ensifera	PCR (fur/cage swab), Direct exam (pelage)	
Rodentolepis (Hymenolepis) spp.	PCR (feces), Direct exam (small intestine)	
Syphacia muris	PCR (feces), Direct exam (cecal contents) Direct exam (perianal tape test)	
FUNGUS		
Pneumocystis carinii[a]	Serology (DBS, serum)	PCR (lung, feces), histology (lung)

DBS, Dried blood spot; *PCR*, polymerase chain reaction.
[a]*Clinical and/or histologic disease is primarily in immune-compromised rats.*
[b]*Antibody to Boone cardiovirus may cross-react with RTV assays.*
[c]*Formerly* Pasteurella pneumotropica *biotype Jawetz and Heyl.*

the presence of the organism. Often, only serum is collected for health monitoring, so samples for confirmatory testing by other platforms may not be possible. In these cases, testing of cohort animals may be warranted and a diagnostic plan to test with different testing platforms should be designed. For example, if a rat is found to be seropositive for a rat parvovirus, additional animals from that colony may be tested by serology and their mesenteric lymph nodes may concurrently be tested by PCR for rat parvovirus. Testing of cohort animals is also warranted in the case where very few animals are seropositive. This scenario may indicate an early outbreak or a false-positive result. If an early outbreak is occurring, cohort animals will have additional time to seroconvert and the percentage of positives should increase.

V. MANAGEMENT OF COLONY DISEASE OUTBREAKS

A. Confirmation and Containment

As discussed earlier, when laboratory testing suggests a change in colony status for a particular agent, it is important to verify the information. Once confidence in the initial lab result is obtained, the positive sample should be tracked back to confirm its origin by comparing the date of testing, or cage or animal identification numbers, etc., to the monitoring schedule and sampling documentation. Based on this information, if an excluded agent appears to be present, the room should be quarantined to prevent further spread throughout the facility while the situation is being further assessed. Scheduled incoming shipments should be diverted to other areas and transfers out of the room should be canceled or approved only after specific risk assessment of where the animals are to be taken and for what purpose. Changes to standard practices that have the potential to affect cross-contamination should be considered, such as the room entry order, the handling and transport of soiled cages, the protective clothing and disinfectants used in the room, and the amount of personnel traffic allowed. A follow-up plan should be implemented to establish whether the agent truly does exist within the room by performing additional confirmatory testing on remaining sentinels or principal animals. It may be useful to draft a generic initial response plan for suspected contamination events in advance, so that these initial steps can be instituted promptly and efficiently.

B. Response Plan

When contamination has been confirmed, a plan of action should be developed by the veterinary and animal facility management groups in concert with others that are affected, such as the research groups holding animals in the area. It is also prudent to include key individuals from the administration or upper management (since there may be a significant fiscal impact) and the institutional animal care and use committee. In some cases, there will be no question what the follow-up response to contamination will be (for example, eradication of the agent), but in others the potential costs and benefits of the available options may need to be considered. Regardless of the decisions made, the plan must be documented and distributed so that the goal is very clear to all involved and the sequence of events and projected timeline are evident.

C. Eradication Options

There are a variety of methods that can be used to eliminate an infectious agent from an area, and careful professional judgment is needed to determine the most appropriate course of action. If the animals are replaceable and the primary consideration is to return the room to normal use, complete depopulation followed by environmental decontamination can be performed. Attempting partial depopulation by removing positive animals (via test and cull) may not be the most productive approach for many rodent colony disease outbreaks due to the large numbers of animals often involved, the delay between exposure and seroconversion (although PCR testing can be used to minimize that concern), and the possibility that the disease will be further spread during the handling and sampling procedures needed to test the entire population. As an alternative approach, if the agent does not establish persistent infection, it may be useful to test and remove the *negative* animals. Fully immune populations should pose little risk of shedding to naïve animals after infection by agents such as coronavirus or Sendai virus. By retaining only previously exposed seropositive animals to reestablish a breeding program, it is possible to produce seronegative offspring (Brammer et al., 1993). A related approach to break the chain of transmission and repopulate an area without determining the serologic status of each individual rat allowed to remain is the cessation of breeding method, also known as *burnout* or *stop-breeding* schemes. By eliminating the introduction of naïve animals from outside and eliminating all internal breeding for a period of time (6–8 weeks is recommended), coronavirus can be

eliminated from a population (Bhatt and Jacoby, 1985; Jacoby and Gaertner, 1994). Reciprocal transfer of soiled bedding between all cages during the early weeks of a burnout period is an optional step that theoretically can help assure that all animals have had equivalent exposure to the agent. Of note, these techniques may not work in immunodeficient rats and should be used with caution in genetically engineered rats.

If rats having a valuable or irreplaceable genotype are involved in an outbreak, there are methods for rederiving the strain. Detailed description of techniques used for rat cesarean section rederivation with or without superovulation have been published (Rouleau et al., 1993; Sharp and LaRegina, 1998). Although the techniques may not be as well established or efficient in rats, superovulation, embryo transfer, and cryopreservation methods similar to those used in mice can be successful in rats and are still being improved upon (Robl and Heideman, 1994; Lambert et al., 2017). More information can be found in Chapter 7 of this text.

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