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CHAPTER

11

Microbiological Quality Control for Laboratory Rodents and Lagomorphs

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OUTLINE

I. INTRODUCTION

Mice (*Mus musculus*), rats (*Rattus norvegicus*), other rodent species, and domestic rabbits (*Oryctolagus cuniculus*) have been used in research for over 100 years. During the first half of the 20th century, microbiological quality control (QC) of lab animals was at best rudimentary as colonies were conventionally housed and little or no diagnostic testing was done. Hence, animal studies were often curtailed and confounded by infectious disease (Mobraaten and Sharp, 1999; Morse, 2007; Weisbroth, 1999). By the 1950s, it became apparent to veterinarians in the nascent field of comparative medicine that disease-free animals suitable for research could not be produced by standard veterinary disease control measures (e.g., improved sanitation and nutrition, antimicrobial treatments) in conventional facilities. Henry Foster, the veterinarian who founded Charles River Breeding Laboratories in 1948 and a pioneer in the large-scale production of laboratory rodents, stated in a seminar presented at the 30th anniversary of the American Association for Laboratory Animal Science, "After a variety of frustrating health-related problems, it was decided that a major change in the company's

Laboratory Animal Medicine, Third Edition
DOI: http://dx.doi.org/10.1016/B978-0-12-409527-4.00011-0

philosophy was required and an entirely different approach was essential." Consequently, he and others developed innovative biosecurity systems to eliminate and exclude pathogens (Allen, 1999). In 1958, Foster reported on the Cesarean-originated barrier-sustained (COBS) process for the large-scale production of specific pathogen-free (SPF) laboratory rodents (Foster, 1958). To eliminate horizontally transmitted pathogens, a hysterectomy was performed on a near-term dam from a contaminated or conventionally housed colony. The gravid uterus was pulled through a disinfectant solution into a sterile flexible film isolator where the pups were removed from the uterus and suckled on axenic (i.e., germ-free) foster dams. After being mated to expand their number and associated with a cocktail of nonpathogenic bacteria to normalize their physiology and prime their immune system, rederived rodents were transferred to so-called barrier rooms for large-scale production. The room-level barrier to adventitious infection entailed disinfection of the room, equipment and supplies, limiting access to trained and properly gowned personnel, and the application of new technologies such as high-efficiency particulate air (HEPA) filtration of incoming air (Dubos and Schaedler, 1960; Foster, 1980; Schaedler and Orcutt, 1983; Trexler and Orcutt, 1999). The axenic and associated rodents mentioned in the COBS process are collectively classified as gnotobiotic to indicate that they have a completely known microflora. By contrast, barrier-reared rodent colonies are not gnotobiotic because they are housed in uncovered cages and thus acquire a complex microflora from the environment, supplies, personnel, and other sources. Instead, they are described as SPF to indicate that according to laboratory testing, they are free from infection with a defined list of infectious agents, commonly known as an 'exclusion' list.

The advances in cell biology, genetics, and analytical methods that coincided with the progress in research animal biosecurity led to discoveries of infections, often by viruses, which although inapparent nonetheless confounded experimental findings by contaminating biological reagents and distorting or modulating *in vivo* and *in vitro* responses dependent on infected host cells (Hartley and Rowe, 1960; Kilham and Olivier, 1959; Riley *et al*., 1960; Rowe *et al*., 1962). There are also documented cases of unrecognized infections altering the phenotype of animal models as has been reported for infections with *Helicobacter* spp. (Horowitz *et al*., 2007; Jurjus *et al*., 2004; Kuhn *et al*., 1993; Kullberg *et al*., 1998; Mombaerts *et al*., 1993; Powrie and Leach, 1995; Strober and Ehrhardt, 1993). In addition, mice and rats were shown to be the reservoir species for zoonotic viruses responsible for disease outbreaks in laboratory personnel exposed to silently infected cell lines or cell line-inoculated rodents (Baum *et al*., 1966; Bhatt *et al*., 1986a; Himan, 1975; Lewis *et al*., 1965; Lloyd and Jones, 1986). Thus, the absence of overt disease was no longer sufficient evidence that animals were either suitable or safe for research. Rather, routine laboratory screening, commonly referred to as health monitoring (HM), was required to detect inapparent infections capable of interfering with research. The traditional HM methodologies used for over half a century have included direct gross and microscopic examinations of animal specimens for parasites and pathology, microbiology consisting of cultural isolation, and phenotypic identification of primary and opportunistically pathogenic bacteria and fungi, and serology, that is, immunoassays of serum or blood samples for specific antibodies formed in response to infections with viruses and several fastidious and invasive microbial pathogens. The newest methodology, molecular diagnostics by polymerase chain reaction (PCR), was introduced in general and to lab animal diagnostics in the mid-1990s; since then, its use in HM has grown, in recent years dramatically, for reasons that will be discussed later (Compton and Riley, 2001; Compton *et al*., 1995; Lipman and Homberger, 2003; Livingston and Riley, 2003; Shek and Gaertner, 2002; Weisbroth *et al*., 1998).

Although rederivation, barrier room production, and HM had become standard practice for commercial rodent breeders by the1970s, a considerable percentage of vendor barrier-reared colonies were reported in the early 1980s still to be infected with a variety of rodent viruses and parasites (Casebolt *et al*., 1988). Moreover, it was apparent that many research establishments were not prepared to maintain the SPF status of commercial rodents, which often became ill or seropositive shortly after being received. Subsequently, major commercial breeders greatly reduced the incidence of barrier room contaminations by more thorough disinfection of rooms prior to stocking, rigorous adherence to standard operating procedures (SOPs), and validation and routine certification of the procedures and equipment for disinfecting supplies. They also discontinued direct room-to-room transfers based on negative HM results, as this practice surely contributed to the inadvertent dissemination of unrecognized pathogens such as the then-novel rodent parvovirus serotypes (Ball-Goodrich and Johnson, 1994; Ball-Goodrich *et al*., 1998; Besselsen *et al*., 1995a, 1996; Jacoby *et al*., 1995; Mckisic *et al*., 1993; Smith *et al*., 1993a) and enterohepatic species of *Helicobacter* (Fox *et al*., 1994, 1996; Ward *et al*., 1994b) identified in the 1990s, and murine norovirus (MNV) first reported in 2003 (Henderson, 2008; Hsu *et al*., 2006; Karst *et al*., 2003; Ward *et al*., 2006). At research institutions, the incidence and prevalence of adventitious infections – where incidence indicates the rate of new contaminations, or outbreaks (e.g., 10 mouse parvovirus (MPV) contaminations/1000 racks/year), and prevalence is the % positive within a time period (e.g., 40% of mice in North America were MNV seropositive in 2012) – were greatly reduced

by switching from housing rodents in uncovered cages, perhaps in barrier rooms, to static and then individually ventilated microisolation cage-level barrier systems shown to be highly effective at excluding and impeding the transmission of adventitious agents (Bohr *et al*., 2006; Hessler, 1999; Lipman, 1999; Sedlacek and Mason, 1977).

While these biosecurity improvements have nearly or completely eliminated once-common pathogens such as Sendai virus from production and research colonies, outbreaks with environmentally stable and highly contagious enterotropic pathogens, such as MPV, mouse rotavirus, and hepatitis virus (MHV), continue to occur (Pritchett-Corning *et al*., 2009). Additionally, transgenic and gene-targeted genetically engineered mouse models for human diseases, which make up a large and rapidly growing proportion of the animals used in research, have been shown often to harbor pathogens such as *Helicobacter* spp., MNV, *Pasteurella pneumotropica*, and parasites largely eliminated from commercial colonies (Carty, 2008; Jacoby and Lindsey, 1998; Pritchett-Corning *et al*., 2009). Likely reasons for this include the recurrent and expanding exchange of genetically engineered mice among research institutions worldwide where QC practices vary and infected rodents may be housed and the initially inadvertent distribution of mice infected with *Helicobacter* spp. and MNV before these agents were recognized and testing for them was available. Thus, genetically engineered mice represent a significant source of adventitious agents capable of confounding experiments, not least of all in genetically engineered models in which these infections have been shown to alter or obscure the effects of genetic modifications, or cause severe and sometimes atypical disease (Compton *et al*., 2003; Franklin, 2006).

The continued occurrence of adventitious infections and the discovery of pathogens underscore the importance of HM results that accurately represent the pathogen status of research animals, but also highlight the limitations of HM and the need for all aspects of microbiological QC including biosecurity with rederivation to eliminate and prevent the dissemination of yet-to-be recognized as well as known pathogens. It is worth noting that this is comparable to the complementary approaches of strict control of production processes and comprehensive quality testing, which are the basis of Current Good Manufacturing Practices (CGMPs) for regulated pharmaceuticals (Tolbert and Rupp, 1989). While the degree of rigor engendered in CGMPs of regulated pharmaceuticals may be excessive for laboratory research, applying the essential aspects of CGMPs to microbiological QC of research animals and biological reagents is a prerequisite for meaningful biomedical research. Insufficient microbiological QC during research can lead to inaccurate findings, the need to repeat experiments, and biological product development setbacks and failures.

In addition to depending on an assay's ability to correctly classify positive and negative samples, the accuracy of microbiological surveillance is contingent on testing specimens that are both representative of the pathogen status of the principal animals and suitable for the assay method. The term 'principals' (or principal animals) refers to the animal populations, or groups, being monitored, whether resident or in quarantine. Residents include colony and study animals housed at a facility. Collecting specimens from microisolation caging systems that are representative of the principals' pathogen status and suitable for the test method is challenging because of the often low prevalence of infection and the shortcomings of soiled bedding sentinel monitoring. PCR testing of non-invasive specimens collected directly from resident and quarantined animals, and of environmental samples not suitable for traditional methodologies is helping address these challenges (Bauer and Riley, 2006; Compton *et al*., 2004c; Henderson *et al*., 2013; Jensen *et al*., 2013; Macy *et al*., 2009, 2011). The process of containing and eradicating adventitious infections is inevitably costly and disruptive to research. Therefore, it is crucial that repeat testing, employing complementary methodologies if possible, be carried out to verify infection of the resident animals before taking remedial measures. Once the occurrence of an adventitious infection has been verified, actions are taken to contain, eradicate, and investigate the contamination with the goal of preventing a recurrence.

In sum, as biomedical research has become more sophisticated, the list of pathogens shown to interfere with research has grown, and hence, research animal SPF specifications have become more rigorous. This chapter reviews the main elements of microbiological QC needed to meet these specifications including biosecurity to eliminate, exclude, and contain pathogens, HM, and the management, eradication, and investigation of contaminations. Although microbiological QC for rodents will be emphasized, the methodologies considered are applicable to laboratory animals in general.

II. MICROBIOLOGICAL QUALITY SPECIFICATIONS

Gnotobiotic animals, whether axenic (i.e., germ-free) or associated with a defined microbiome consisting of a few nonpathogenic bacteria, make up a small fraction of the animals used in research; however, their usage is likely to increase with the growth of research into the profound influences and diverse effects of the microbiome on human health and the experimental responses of research models (Bech-Nielsen *et al*., 2012; Friswell *et al*., 2010; Grada and Weinbrecht, 2013). As already mentioned, most lab animals are referred to as SPF to

indicate that they have been shown by HM to be free of pathogens on an exclusion list.

Exclusion lists for rodents, rabbits, and other common lab animal species have been substantially harmonized throughout the developed world due to the efforts of lab animal science organizations (Guillen, 2012; Nicklas, 2008; Nicklas *et al*., 2002), and the globalization of biomedical research. In addition, competition for customers encourages vendors to offer SPF animals free of newly discovered pathogens and diagnostic laboratories to develop tests for those pathogens (Shek, 2000). The exclusion lists for SPF mice and rats are more extensive than those for rabbits and other common lab animal species for a number of reasons. First, as mice and rats account for the vast majority of animals used in research, it stands to reason that more is known about their indigenous pathogens than those of other less used lab animal species. The diversity of inbred, and naturally and genetically engineered immunodeficient mutant rodent strains highly susceptible to infectious disease (Compton *et al*., 2003; Franklin, 2006), in conjunction with sensitive immunoassay methods (Smith, 1986b) and advances in molecular genetics (Compton and Riley, 2001), has contributed to the discovery and characterization of rodent pathogens (Fox *et al*., 1994; Ward *et al*., 1994a) found to be the cause of ubiquitous, inapparent infections of laboratory rodent colonies (Hsu *et al*., 2006; Shames *et al*., 1995). In addition, the predominance of murine rodent research models has provided strong incentives for diagnostic laboratories and vendors to develop and offer specific serologic and PCR assays for viral and other fastidious microbial pathogens – not amenable to detection by direct microscopic examination or cultural isolation – soon after their discovery. By contrast, commercial vendors and diagnostic laboratories have had little demand from the research and lab animal medicine communities to provide routine serologic and PCR testing for rabbit viruses recognized decades ago, such as lapine parvovirus (Matsunaga and Matsuno, 1983) (which sequencing has recently shown to be a bocavirus (personal communication, K Henderson)), rabbit enteric coronavirus (Deeb *et al*., 1993; Descoteaux and Lussier, 1990; Descoteaux *et al*., 1985), and leporid herpesvirus 2 (Matsunaga and Yamazaki, 1976). Finally, rederivation by hysterectomy or embryo transfer (ET) to eliminate all exogenous pathogens is the standard practice for SPF mice and rats, but not for other species.

SPF exclusion lists for mice and rats have included all known exogenous viruses regardless of virulence because as obligate intracellular parasites, viruses are inherently invasive; furthermore, even noncytopathic viral infections have been shown to alter the metabolism of host cells (Oldstone *et al*., 1982). Strict adherence to the dogma of excluding all exogenous viruses from SPF mice and rats, however, has become impractical at many research institutions where asymptomatic MNV infections, primarily in genetically engineered mice, are considered to be too widespread to be eliminated. Leading-edge molecular genetic techniques have recently uncovered a murine astrovirus (Farkas *et al*., 2012), possibly more common in mice than MNV, and will surely find additional prevalent viruses that have so far eluded detection because they, like MPV, MNV, and murine astrovirus, are highly host-adapted and by and large apathogenic even for immunodeficient hosts. As noted, viral exclusion lists for rabbits and other lab animal species are less comprehensive than for murine rodents.

Ectoparasites, helminths, pathogenic protozoa, bacteria, and fungi are part of the exclusion lists of all SPF animal species. The pathogenic bacteria and fungi excluded for SPF animals are mainly distinguished from commensal and autochthonous (i.e., indigenous) organisms by their ability to cross anatomic and biochemical barriers to establish themselves in niches devoid of other microorganisms such as the lower respiratory and urogenital tracts, internal organs, and intracellularly (Casadevall and Pirofski, 2000; Council, 2009; Merrell and Falkow, 2004). Pathogenicity is not necessarily an immutable characteristic of the microbial species as normally commensal microbes such as *Escherichia coli* have been transformed into pathogens through the acquisition of virulence genes transferred from other bacteria in mobile genetic elements such as plasmids, phages, and transposons (Dobrindt *et al*., 2004).

Microbes are classified as primary pathogens if they can cause disease in immunocompetent hosts. Examples include *Salmonella*, *Mycoplasma pulmonis*, *Helicobacter hepaticus*, and *Clostridium piliforme* (the etiology of Tyzzer's disease). Opportunistic pathogens such as *Pseudomonas aeruginosa*, β-hemolytic streptococci, *Staphylococcus aureus*, and *Pneumocystis* fungi cause disease mainly in immunocompromised hosts, whether (1) immunosuppressed by irradiation or chemotherapy (Bosma *et al*., 1983; Cryz *et al*., 1983; Flynn, 1963; Homberger *et al*., 1993; Rosen and Berk, 1977; Waggie *et al*., 1988; Walzer *et al*., 1989; Weir *et al*., 1986; Weisbroth *et al*., 1999) or (2) inherently immunodeficient, such as athymic nude and severe combined immunodeficient (SCID) mice (Bosma *et al*., 1983; Clifford *et al*., 1995; Dole *et al*., 2013b; Henderson *et al*., 2012; Pantelouris, 1968; Ward *et al*., 1996). For the most part, only primary microbial pathogens are included in SPF exclusion lists for immunocompetent animals. Opportunists are added, chiefly by commercial vendors, to lists for immunodeficient and genetically engineered mutant lines. Because it is not unusual for opportunists such as *S. aureus* to cause disease in standard (i.e., nongenetically engineered) immunocompetent strains of rodents (Besch-Williford and Franklin, 2007), which are often used in rederivation and breeding schemes for genetically engineered

lines, the demand for standard, immunocompetent rodent strains and stocks free of opportunistic as well as primary pathogens has increased. This subset of SPF animals has been referred to as SOPF for specific opportunistic pathogen-free.

To summarize, the infectious agents on SPF exclusions lists are determined by general and institution-specific criteria. In general, the SPF exclusion lists of mice and rats are more comprehensive than those of less popular animal species because more of the indigenous murine viral and host-adapted microbial pathogens have been identified and studied; serologic and PCR assays are made available for murine pathogens soon after their discovery; and rederivation to eliminate all exogenous pathogens from SPF mouse and rat populations is standard practice. The SPF exclusion lists of all species typically contain ectoparasites, endoparasites, and microbes classified as primary pathogens as well as viruses; vendors often add opportunistic pathogens for immunodeficient and genetically engineered mutant murine models.

Complying with consensus SPF standards can be problematic at an institution if the prevalence of infection is high or barrier systems and practices are inadequate to prevent adventitious infections from recurring and spreading. Many research-intensive academic institutions have decided that the benefits of eliminating prevalent infections with recently recognized agents such as MNV and *Helicobacter*, which rarely produce disease and/or have been endemic to their research colonies for many years, are outweighed by the disruption to research and costs of doing so. However, the elimination (and exclusion) of prevalent pathogens and compliance with consensus SPF standards reduces the risk that a pathogen will infect additional colonies and interfere with research, and simplifies the exchange of animal models and collaborative studies with other investigators and institutions.

III. BIOSECURITY

Lab animal biosecurity consists of all measures taken to eliminate, exclude, contain, and eradicate adventitious infections. Containment and eradication will be discussed further in the section on Outbreak Management and Investigation.

A. Elimination

1. Rederivation

Rederivation of SPF lab animal stocks from those that are harboring pathogens is widely regarded as the most dependable approach for eliminating infections with unrecognized as well as known pathogens. In association with advances in assisted reproductive technologies, ET into SPF pseudopregnant recipient females has supplanted nursing of Cesarian section-originated pups by gnotobiotic or SPF foster mothers as the gold standard for rederivation (Suzuki *et al.*, 1996; Van Keuren and Saunders, 2004). Cesarean rederivation is considered to be less reliable than ET because vertical transmission of infections to fetuses has been demonstrated for viruses (Barthold *et al.*, 1988; Jacoby *et al.*, 2001; Katami *et al.*, 1978; Lehmann-Grube, 1982) and for bacteria capable of colonizing the uterus (Brown and Steiner, 1996; Matsumiya and Lavoie, 2003; Reyes *et al.*, 2000, 2004; Ward *et al.*, 1978). Vertical transmission by ET is unlikely because the zona pellucida that surrounds embryos and oocytes excludes pathogens (Peters *et al.*, 2006) and the risk of infecting the recipient females is minimized by extensive washing of the embryos. In a recent study, ET from MPV-infected SCID mice eliminated the infection (Besselsen *et al.*, 2008b). Other advantages of ET *vis-à-vis* Cesarean rederivation are that it can be combined with other artificial methods like *in vitro* fertilization (IVF) and intracytoplasmic injection of sperm (ICSI) to overcome the reproductive defects that are common in aged and in naturally and genetically engineered mutant mice (Suzuki *et al.*, 1996). Embryos, ova, and sperm (i.e., germplasm) can be cryopreserved to reduce *per diem* costs, save valuable cage space, and assure that unique genetically engineered strains can be rederived at any time and, hence, are never lost. Finally, ET is more efficient because embryo donors are usually superovulated resulting in more offspring per female than are obtained from natural matings (Mazur *et al.*, 2008).

Cesarean rederivation, however, is still extensively used by commercial vendors for standard rodent strains and stocks, and may be preferable to ET for certain genetically engineered rodent lines with fertility issues or whose embryos exhibit low viability in culture. Although less dependable than ET, Cesarean rederivation is highly effective as supported by the observation at Charles River Laboratories that thousands of isolator-maintained rodent colonies, originated by Cesarean rederivations carried out over many years, were without exception free of murine parvoviruses, *Helicobacter* spp. and MNV when these agents were first recognized (W. Shek, unpublished). Neonatal transfer of mouse pups within several days of parturition to SPF foster mothers, after being immersed in disinfectant, has been reported to successfully eliminate a variety of common mouse pathogens including MPV, MHV, MNV, and *Helicobacter* spp., and has the advantages in comparison with Cesarean section of being less expensive and not requiring that valuable breeders be euthanized (Huerkamp *et al.*, 2005; Lipman *et al.*, 1987; Truett *et al.*, 2000; Watson *et al.*, 2005).

Rederivation with rare exception requires pathogen-free animals of the same species to receive embryos or nurse offspring, and to associate progeny with a normal autochthonous microbiome. For mice and rats, this has been accomplished over many years by overcoming various technical hurdles such as (1) hand rearing and meeting the unique nutritional needs of germfree animals; (2) development of defined cocktails of non-pathogenic bacteria to colonize the intestinal tract of ex-germfree animals in order to resist colonization by pathogenic microbes, to normalize host physiology, and to stimulate the immune system; and (3) maintaining rodents in isolators or microisolation cages on sterile supplies to exclude all exogenous microbes from axenic and defined flora animals (Foster, 1980; Schaedler and Orcutt, 1983; Trexler, 1983; Trexler and Orcutt, 1999). The production of defined flora pathogen-free animals is uncommon for other lab animal species but has been described for rabbits (Boot *et al*., 1985, 1989b; Yanabe *et al*., 1999) and guinea pigs (Boot *et al*., 1989a). Cross-fostering offspring onto SPF animals of a different species has been attempted when SPF animals of the same species are not available. In a successful example, *H. hepaticus* was eliminated from Mongolian gerbils by Cesarean rederivation with cross-fostering of the offspring onto SPF mice and rats. The percent survival of rederived gerbils was higher for those nursed by mice (Glage *et al*., 2007).

2. *Alternatives to Rederivation*

Although not considered to be as dependable as rederivation, chemotherapeutic treatments and test-and-cull procedures (Macy *et al*., 2011; Smith, 2010) are increasingly employed to eliminate, or eradicate, adventitious infections from the colonies of unique genetically engineered mutant mouse lines that may not be available from commercial sources and hence are difficult to replace. Microisolation caging systems have contributed substantially to the feasibility and efficacy of these alternatives to rederivation by keeping the prevalence of infection low (Bohr *et al*., 2006), minimizing the level of environmental contamination, and thereby reducing the chance of reinfection. Nevertheless, rederivation and eventual cryopreservation of unique mutant animal models are considered crucial to ensuring their survival, availability, and freedom from known and yet-to-be recognized pathogens.

Chemotherapy is mainly employed to cure or prophylactically treat pinworm and mite infestations of quarantined and resident rodents housed in microisolation cages. The most effective and frequently administered antiparasite medications are the avermectins (e.g., ivermectin and selamectin) and benzimidazoles (e.g., fendbendazole); they are normally added to the diet or drinking water, or applied topically (Pritchett and Johnston, 2002; Ricart Arbona *et al*., 2010b). Therapeutic doses of these drugs, however, may cause toxicity as has been demonstrated for ivermectin given to rodents with compromised blood–brain barriers because of young age (Lankas *et al*., 1989; Skopets *et al*., 1996), genetic background (Jackson *et al*., 1998; Lankas *et al*., 1997), or genetically engineered mutations (Schinkel *et al*., 1994, 1997). Antibiotic treatments have been shown to eliminate infections with host-adapted bacterial pathogens, such as *P. pneumotropica* (Goelz *et al*., 1996; Matsumiya and Lavoie, 2003) and *H. hepaticus* (Foltz *et al*., 1996; Kerton and Warden, 2006; Russell *et al*., 1995), which do not survive for long *ex vivo* and, therefore, are unlikely to reinfect hosts after treatment is stopped. Even when effective, however, drug therapies may be too expensive or laborious to be practical, particularly when treating large numbers of animals. However, the labor and cost of large-scale chemotherapy have been greatly reduced by using commercially available medicated diets for treating parasite infestations (Ricart Arbona *et al*., 2010b) and *Helicobacter* infections (Kerton and Warden, 2006; Whary and Fox, 2006).

Unlike the parasite infestations and bacterial infections just mentioned, viral infections cannot be treated; however, low-prevalence viral infections can be eradicated from rodents housed in microisolation cages by test-and-cull procedures discussed further in Section V. Briefly, breeding and the introduction of naive animals to the infected room(s) and rack(s) are stopped and 100% of microisolation cages are tested at regular intervals by serology, PCR, or both. Positive cages are culled and testing at regular intervals is continued for a limited period until either the remaining cages are repeatedly negative or the rack(s) are depopulated and resident animals euthanized, relocated, and/or rederived. Test-and-cull procedures have been successfully utilized to eradicate outbreaks with *Helicobacter* spp. (Beckwith *et al*., 1997; Fermer *et al*., 2002; Hodzic *et al*., 2001; Mahler *et al*., 1998; Shames *et al*., 1995), murine parvoviruses (Bauer and Riley, 2006; Macy *et al*., 2009, 2011), MHV (Compton *et al*., 2004a; Manuel *et al*., 2008), MNV (Manuel *et al*., 2008), and murine rotavirus (A.L. Smith, unpublished).

Another alternative to rederivation applied historically to nonpersistent infections of immunocompetent hosts with enveloped viruses (e.g., Sendai virus and sialodacryoadenitis virus (SDAV)) is to break the cycle of infection by instituting a 6- to 8-week moratorium on breeding and the introduction of naive animals (Bhatt and Jacoby, 1985). During this period, it was expected that all animals in the colony would recover from infection and stop shedding virus, and that the excreted virus would quickly become noninfectious due to the environmental lability of these agents. Historical success was enhanced by the small size of the affected population, and contemporary mouse housing rooms are likely to

contain 700–800 cages. A time-efficient alternative to a breeding moratorium is to start a new colony with seropositive, noncontagious breeders (Brammer *et al*., 1993). It is worth emphasizing that breaking the cycle of infection, commonly called 'burnout', is not recommended in contemporary rodent colonies due to the increasing proportion of genetically modified rodents in populations and the frequently unknown immune competence and atypical response to infection of such animals. The relevance of burnout has been further eroded by the virtual disappearance from research colonies of infections with enveloped respiratory viruses to which this approach was applicable. Most of the enteric viruses that continue to be present in research colonies today are nonenveloped and hence environmentally stable, and persist for prolonged or indefinite periods in the tissues of hosts, including those that are immunocompetent and seropositive. MNV, the most prevalent pathogen of mice, is shed indefinitely (Hsu *et al*., 2006; Manuel *et al*., 2008). If despite these caveats breaking the cycle of infection is attempted, confirmation of eradication is best achieved by PCR or by serosurveillance of sentinels instead of the colony offspring that will likely have maternal antibodies.

B. Exclusion

To exclude pathogens, research animals are maintained behind sanitized and disinfected room- to cage-level barriers provided with filtered air and disinfected supplies and equipment. Biosecurity procedures pertaining to personnel, animal maintenance, pest control, disinfection, and so forth should be regularly reviewed and revised to further reduce the risks associated with potential sources of adventitious infection.

1. Barrier Systems

Barrier rooms, with animals kept in open cages, continue to be employed by commercial breeders for the efficient, large-scale production of immunocompetent rodents and rabbits. Because opportunistic pathogens are not reliably excluded from barrier room colonies (Blackmore and Francis, 1970; Fallon *et al*., 1988; Geistfeld *et al*., 1998), the production of mice and rats that need to be SOPF, in particular known and potentially immunodeficient mice and rats, has been transferred from barrier rooms to flexible-film and semirigid isolators, and filter-covered microisolation cages that provide a higher degree of biosecurity.

Although the effectiveness of filter-covered cages for excluding and controlling the spread of infections had already been demonstrated for mouse rotavirus in1958 (Kraft, 1958), this cage-level barrier strategy did not become popular until the 1980s when commercial microisolation caging systems were introduced. The first microisolation cages were referred to as 'static' to indicate that they relied on passive ventilation. As the temperatures, humidity levels, and noxious gas concentrations (such as CO_2 and NH_3) in static cages were found to be significantly elevated in comparison with room levels, actively ventilated microisolation caging systems were developed to enhance the cage microenvironment (Les, 1983) and to allow for higher animal densities with fewer cage changes. Moreover, ventilated systems can be exhausted directly into the facility HVAC to improve the room environment and can be run under a negative pressure differential for pathogen containment. To maximize biosecurity, microisolation cages should only be opened in a HEPA-filtered air laminar flow change stations or biological safety cabinets by technicians wearing personal protective equipment (PPE) and following sterile technique when manipulating animals. Microisolation cages are often located in barrier rooms to provide a further obstacle to microbial contamination (Hessler, 1999; Lipman, 1999; Sedlacek and Mason, 1977; Trexler and Orcutt, 1999). Today, microisolation caging systems house the majority of rodents at research-intensive academic, biotechnical, and pharmaceutical institutions. There is little doubt that the effectiveness of these cage-level barrier systems at excluding and impeding the spread of infections, and their tolerance of systemic deficiencies and operator errors, has played a major part in lowering the frequency of microbial contaminations at biomedical research facilities and keeping the prevalence of infection low following an outbreak (Macy *et al*., 2011; Shek, 2008; Whary *et al*., 2000a). The challenges that this low prevalence presents to obtaining test results that accurately represent the pathogen status of principal animals (i.e., the resident colony or study animals, or the quarantined animals being monitored) will be discussed in Section IV.

2. Mitigating Risks from Sources of Infection

Irrespective of the barrier system, successful exclusion of pathogens from rodent colonies depends on an understanding of the chain of adventitious infection, which comprises reservoirs, sources, and modes of transmission (Fig. 11.1), and mitigating the risks associated with sources of infection (Table 11.1). The reservoir, or ecological niche, of a microorganism can be an animal species or the environment (Brachman, 1996). For example, the reservoir for lymphocytic choriomeningitis virus (LCMV) is the wild mouse (Lehmann-Grube, 1982), whereas *Listeria monocytogenes* is found in various avian and mammalian species as well as throughout the environment (Broome *et al*., 1998). The source of an organism for transmission to a susceptible host is not necessarily the same as its reservoir. The source of *L. monocytogenes* for an SPF colony might be food or bedding that was contaminated by carrier animals or the environment. The distinction between reservoir and

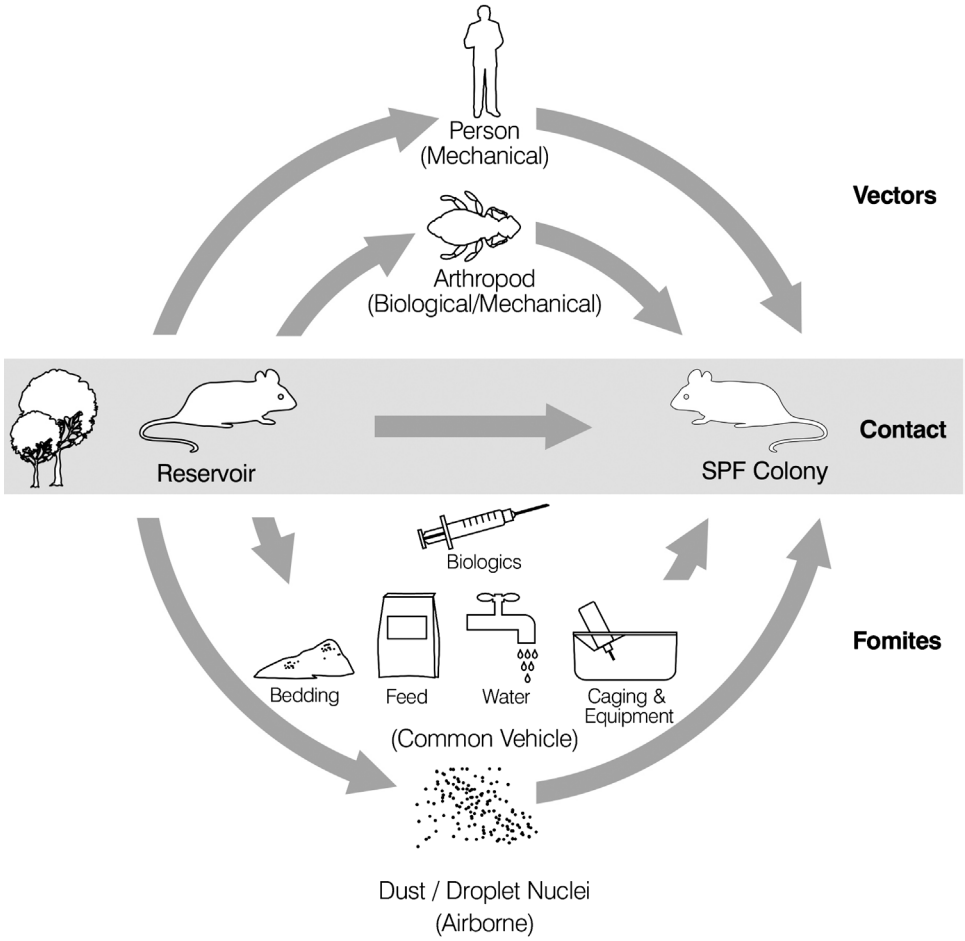

FIGURE 11.1 Chain of adventitious infection for laboratory rodents. The reservoir, or ecological niche, of a microorganism can be an animal species or the environment. The principal reservoirs of adventitious infection for SPF rodents are other rodents of the same or related species. Infection can be transmitted directly from animal to animal or indirectly by an inanimate vehicle, also termed a fomite, or a vector.

source is important in the case of lab animal biosecurity because, in general, it is much more practical to control a pathogen's source than its reservoir.

Given that most pathogens are obligate parasites with a limited host range, it stands to reason that infected animals of the same or related species are the principal reservoirs of adventitious infection for SPF lab animal populations. Infection can be transmitted directly from animal to animal or indirectly by an inanimate vehicle, also termed a fomite, or an animate vector.

Contact transmission is vertical when it takes place *in utero* or at birth, or horizontal if it occurs postpartum through the transfer of droplets or by intimate contact, as exemplified by venereal diseases. Most pathogens of rodents and rabbits are efficiently transmitted by direct contact (Parker and Reynolds, 1968; Shek *et al*., 1998; Thigpen *et al*., 1989; Yang *et al*., 1995). Lactate dehydrogenase-elevating virus (LDV) is a notable exception that is mainly spread by parenteral injection of naive mice with transplantable mouse cell lines (Collins and Parker, 1972; Nicklas *et al*., 1993; Riley, 1974). LDV has been recently shown to contaminate a basement membrane matrix used by tumor biologists and the cell line from which it was derived (Carlson Scholz *et al*., 2011; Liu *et al*., 2011; Nagaoka *et al*., 2010). The efficacy of Cesarean rederivation shows that vertical transmission of rodent pathogens is uncommon although it has been demonstrated to occur for various viruses in immunodeficient or acutely infected dams (Barthold *et al*., 1988; Jacoby *et al*., 2001; Katami *et al*., 1978; Lehmann-Grube, 1982), for LCMV in enzootically infected mice (Lehmann-Grube, 1982) and for bacterial pathogens capable of colonizing the uterus (Brown and Steiner, 1996; Matsumiya and Lavoie, 2003; Reyes *et al*., 2000, 2004; Ward *et al*., 1978).

Fomite transmission can be airborne, referring to the spread of contaminated droplet nuclei (i.e., the residue of dried droplets) or dust for a distance of more than several feet (Brachman, 1996), or by way of common vehicles such as food, water, bedding, and equipment. Aerosol transmission of enveloped respiratory viruses to

TABLE 11.1 Mitigating Risks for Sources of Infection

Transmission	Source	Risk mitigation
Direct contact	Wild or escaped rodents	Rodent proof construction Pest control program Barrier maintenance
	Imported animals	HM records from source Quarantine with HM Rederivation
	Personnel	Gowned (PPE) Animals manipulated in hood/ isolator Restricted access Pet policy
Fomite	Room/equipment surfaces	Chemical disinfection Manipulate animals in hood
	Food, bedding, supplies	Autoclaving, gamma irradiation
	Airborne	HEPA filtration Air pressure differential
	Waterborne	Filtration, chlorination, UV irradiation
Vector	Insects	As for wild rodents
	Personnel	As above
Inoculation of biologic	All biologics	Testing: PCR for rodent pathogens, sterility/bioburden, mycoplasma
	Cell line	Bank cells
	Other biologics	Physical or chemical disinfection

sentinels has been demonstrated in ventilated microisolation caging systems by housing sentinels in cages with unfiltered exhaust air from infected colony cages (Compton *et al.*, 2004c); however, its overall significance to the spread of disease in laboratory rodents has been diminished based on the eradication of rodent respiratory pathogens and the predominance of covered microisolation cages, which minimize the opportunity for airborne spread. By contrast, common vehicles continue to be highly important for two reasons. First, soiled bedding is usually the primary or only source of infection for microisolation cage sentinels, and studies have shown that infections with host-adapted bacteria, enveloped viruses, and other organisms that are unstable *ex vivo* are not transmitted efficiently or at all in soiled bedding (Artwohl *et al.*, 1994; Compton *et al.*, 2004c; Cundiff *et al.*, 1995; Dillehay *et al.*, 1990; Henderson *et al.*, 2013; Ike *et al.*, 2007; Thigpen *et al.*, 1989). Fortunately, soiled bedding has been reported to transmit many of the enteric viruses and microbial pathogens that are common today (Compton *et al.*, 2004c; Grove *et al.*, 2012; Livingston *et al.*, 1998; Manuel *et al.*, 2008; Perdue *et al.*, 2007; Smith *et al.*, 2007; Whary *et al.*, 2000b), provided that the sentinels are exposed to a sufficient dose of the pathogen to infect them (Smith *et al.*, 2007). Second, evidence supports food and bedding as important sources of the multi-institutional MPV and mouse rotavirus outbreaks that have repeatedly occurred over the years. For instance, at institutions where some colonies were given gamma-irradiated or autoclaved food and bedding and others were not, outbreaks were by and large restricted to the colonies receiving non-disinfected supplies (Reuter *et al.*, 2011). Furthermore, a noticeable drop in the incidence of MPV and mouse rotavirus outbreaks has been observed at vivaria that have switched to disinfected (typically γ-irradiated) food and bedding. A recent report linked MPV-1 and MPV-2 outbreaks to medicated diet that had not been disinfected by showing that the locations of mice experiencing the outbreaks matched the distribution of the untreated medicated diet (Watson, 2013). In another recent study, concomitant mouse rotavirus outbreaks at five institutions were related to the use of non-disinfected bedding from a common source by showing that the genetic sequences of mouse rotaviruses from the different institutions were identical (Dole *et al.*, 2013a).

Vectors can be biological, i.e., essential to the life cycle of the pathogenic organism, or mechanical (Brachman, 1996; Cohen, 1998; Prince *et al.*, 1991; Waggie *et al.*, 1994). Arthropod vectors play a minor role in the transmission of rodent pathogens. Lice are known biological vectors for the erythrocyte parasites *Eperythrozoon coccoides* and *Mycoplasma haemofelis*, formerly *Haemobartonella muris* (Neimark *et al.*, 2002), of mice and rats, respectively (Hildebrandt, 1982), but these louse vectors and rickettsial parasites are no longer encountered in laboratory mice (Jacoby and Lindsey, 1998), although they still may be common in pet and wild mouse population. Both insects and people have been incriminated as mechanical vectors for adventitious viral infections (Ishii *et al.*, 1974; Tietjen, 1992). To summarize, adventitious infection occurs when an etiologic agent is accidentally transmitted from its reservoir, most often animals of the same species, into an SPF animal colony by direct animal-to-animal contact or indirectly through a fomite or vector.

a. Animals

As mentioned, the most likely animal reservoir of infection for SPF rodent colonies are other rodents, whether wild or feral (i.e., escaped), housed nearby in the same facility, or imported. Wild rodents have been shown to carry a variety of pathogens that contaminate SPF facilities (Behnke, 1975; Bhatt *et al.*, 1986b; Chabe *et al.*, 2010; Childs *et al.*, 1989; Ike *et al.*, 2007; Parker

et al., 2009; Singleton *et al*., 1993; Skinner *et al*., 1977; Smith *et al*., 1993b). The risk of their contaminating an SPF colony is expected to increase when a rodent control program is not in place or the structural barriers to entry are inadequate (Lussier *et al*., 1988). Pest control services are best provided by a reputable and licensed commercial vendor. Animal facilities should be constructed and maintained so that potential nest areas and routes of ingress or egress are not present. All holes and cracks in the facility should be sealed. No matter how careful the oversight of construction projects, there may be unanticipated problems. A relatively new housing facility very suddenly revealed evidence of increased wild/feral mouse activity. Doors that were to be completely sealed were found to have small holes on the bottom surface and mice were breeding in the Styrofoam filler that was used in the doors for noise abatement (A.L. Smith, unpublished). A widespread fur mite outbreak in that vivarium about 1 year later was attributed to the earlier mouse infestation and necessitated treatment of all mice in the facility at a cost of $102,000. Trapping devices should be used to detect and eliminate loose rodents. Those that are captured alive should be identified as to species, handled as if they were infected, anesthetized, bled for serology, and examined for internal and external parasites prior to euthanasia. Whether loose rodents are captured dead or alive, specimens from them (e.g., tissues, feces, and swabs) are suitable for testing by microbial PCR as an adjunct or alternative to traditional diagnostic methodologies. Food, bedding, and garbage attract loose rodents and therefore should be stored off the floor in a secure area in sealed containers (Hoddenbach *et al*., 1997; Small, 1983). Vivaria that are located in multiuse buildings, frequently including offices and common areas at academic institutions, are at high risk because food is often present and may not be cleared until the next day after late social events. Sealed trash containers should be used in these situations to decrease the likelihood of rodents having access to food.

The risk of introducing pathogens through the transfer or importation of animals from another investigator or institution is affected by a variety of factors that are not mutually exclusive. These include the type of animals being imported, the source institution's microbiological QC program, the prevalence and incidence of infections, and the method of transportation.

It is generally the case that genetically engineered mutant mice produced at academic institutions have a high prevalence of infection with MNV, *Helicobacter* spp., *P. pneumotropica*, and parasites (Carty, 2008; Jacoby and Lindsey, 1998; Pritchett-Corning *et al*., 2009). By contrast, the risk of unexpectedly introducing pathogens with rodents and rabbits from large commercial breeders is minimal. Commercial vendors that have historically low rates of contamination, transportation dedicated to SPF animals, and a track record of reliability are usually put on an institution's 'approved vendors list', which exempts their animals from quarantine. At large, research-intensive academic institutions, the vast majority of rodents acquired by scientists are likely to be procured from 'approved' commercial vendors. However, while the absolute number of animals is much smaller, these same scientists frequently need to procure unique strains of rodents from colleagues at academic institutions or biotech or pharmaceutical companies. These are so-called nonapproved vendors and most institutions have policies and programs that provide quality control testing of these animals prior to their release into the general resident population. Among the steps taken to mitigate risks associated with importing these rodents are (1) review of health reports from the source colony (often 1 year's worth of data may be requested) to ensure the animals are not likely to carry an agent excluded by the recipient institution; (2) quarantine of the newly arrived rodents in a remote area that provides barriers to transmission of agents to colony animals; and (3) a program to monitor the health of the animals prior to release from the quarantine area. Some quarantine facilities house rodents in static microisolation cages, some on ventilated racks, some in individually ventilated cubicles, and, although more rarely, some in semirigid isolators. Used properly, any of these housing modalities can provide effective isolation. Isolators are labor-intensive to use and service but provide very good isolation.

During the quarantine period, the principal animals being monitored for infection ideally should be cohoused with SPF sentinels for part or all of the quarantine to maximize the chance of sentinel infection. The quarantine typically lasts 4–8 weeks to allow sufficient time for sentinels to get infected and seroconvert. Alternatively, the need for sentinel testing can be bypassed and the time in quarantine reduced to about 2 weeks by PCR testing of noninvasive, *ante mortem* specimens (e.g., feces and swabs of the fur, perianal region, and oral cavity) collected directly from the principals within a week of their arrival. By reducing the time in quarantine, direct PCR testing of principals makes more efficient use of quarantine space, decreases the chance of cross-infection among imported cohorts, and affords investigators quicker access to their animals. Most importantly, as shown in a recent study, direct PCR testing of principals is more sensitive than indirect sentinel screening (Henderson *et al*., 2013), which is not unexpected as PCR typically detects pathogen levels well below the infectious dose for animals and cell culture (Bauer *et al*., 2004; Bauer and Riley, 2006; Blank *et al*., 2004).

One reason to monitor the health of the imported mice is to ensure that they were not inadvertently exposed to any excluded pathogens during transport. Whereas approved vendors usually employ dedicated

trucks that carry animals of known health status, nonapproved source rodents are normally transported by commercial carriers and private couriers whose quality control practices cannot be monitored. Also, a proportion of the nonapproved source animals come from laboratories with HM programs of unknown quality, so checking the health status of the imported animals is a good insurance policy and protects the health of the destination colony.

b. Supplies and Equipment as Common Vehicles

The risk of fomite transmission may be reduced by using physical and chemical processes to sterilize or disinfect equipment and supplies. Sterilization is the elimination or inactivation of all microorganisms, whereas disinfection is less complete. For example, a disinfection process might destroy vegetative bacteria but not bacterial spores (Block, 1991). Supplies for gnotobiotic colonies must be sterilized, whereas disinfection, or pasteurization, generally suffices for supplies being transferred into an SPF area (Foster, 1980; Foster *et al.*, 1964; Trexler, 1983). Rational selection of a disinfection or sterilization process is aided by knowledge of the process's mechanism of action and the physicochemical characteristics of the microorganisms to be eliminated. In general, bacterial spores, free-living stages of parasites (e.g., pinworm eggs and protozoan cysts), and hydrophilic nonenveloped viruses (e.g., MPV) are resistant to inactivation (Ganaway, 1980; Hoover *et al.*, 1985; Leland, 1991; Prince *et al.*, 1991; Russell, 1991; Van Der Gulden and Van Erp, 1972). The best method for disinfection is also determined by the process's applicability to a particular medium (e.g., air, food, water, and surfaces), hazards (including corrosive properties), and the toxicity of treatment, ease of application, and cost. The efficacy of disinfection procedures and equipment should be validated and routinely monitored using biological, chemical, and/or physical indicators (Russell, 1992).

Physical Processes of Disinfection Physical processes of disinfection, such as autoclaving and electromagnetic irradiation, are the treatments of choice for food and bedding. By contrast to chemical disinfection, these methods do not leave a residue or by-products that may be toxic for or cause physiologic changes in animals (Hermann *et al.*, 1982). Raw materials used in the preparation of animal feed and bedding frequently have a high bacteria count. The heating of food to 75–80°C during pelleting substantially reduces the bacterial count but is not sufficient to inactivate thermostable pathogens. In addition, food and bedding may become recontaminated after processing (Clarke *et al.*, 1977). Therefore, they should be sterilized or pasteurized for gnotobiotic or SPF rodent colonies, respectively. As mentioned, this has traditionally been accomplished by autoclaving (i.e., saturated steam heat) or gamma irradiation. In comparison with gamma irradiation, autoclaving is less expensive but causes a greater reduction in the nutritional value of food (Ferrando *et al.*, 1981). Another drawback of autoclaving is the difficulty in achieving uniform steam penetration and temperature throughout a load (Small, 1983). Presterilization vacuum cycles help preserve the nutritional value of food by promoting rapid and uniform steam penetration, which allows autoclave times to be kept short (Foster *et al.*, 1964; Maerki *et al.*, 1989).

Gamma radiation, usually emitted from a cobalt-60 source, is a type of ionizing radiation. Although ionizing irradiation has a variety of physical and biochemical effects, it mainly renders microorganisms nonviable by causing breakage in their nucleic acid (Silverman, 1991). Ultraviolet (UV) radiation (210–328 nm), which does not possess sufficient energy to cause ionization, also inactivates microorganisms by damaging their DNA but does not cause DNA breakage. Instead, UV irradiation produces thymine and other pyrimidine dimers. As one might expect, the bactericidal activity of UV irradiation is maximal near the peak of DNA absorption, which is 260 nm (Russell, 1991). Gamma radiation passes through solid objects; by contrast, UV radiation does not and therefore is effective only for disinfection of surfaces and drinking water. UV inactivation of microbes in drinking water is reduced as the UV light source loses intensity or becomes dirty and by the presence of particles and dissolved organics in the water (Sobsey, 1989). Nonetheless, UV irradiation is an attractive option for water disinfection because it is virucidal and, in contrast to chlorination, does not convert organic precursors into potentially carcinogenic trihalomethanes (Flood, 1995).

The radiosensitivity of organisms has been shown to correlate with genome volume and the ability of the organism to repair DNA damage (Silverman, 1991). This is the reason why comparatively small viruses, such as parvoviruses, are highly resistant to UV and gamma irradiation (Hanson and Wilkinson, 1993), as are bacterial spores, protozoan cysts, and vegetative bacteria with highly efficient DNA repair capabilities (Russell, 1991). Accordingly, irradiation should not be relied on as the sole treatment for sterilization of supplies intended for gnotobiotic rodents.

Filtration is the process most often employed to remove microbes from air and water (Denyer, 1992; Levy and Leahy, 1991). Depth filters entrap and adsorb, whereas membrane filters exclude particles according to pore size. They have high 'dirt-handling' capacity, and therefore they are used for HEPA filtration and for clarification of particle-laden liquids. Because depth filters have no meaningful pore size, they are given nominal ratings to indicate the efficiency with which they retain particles of a particular size. The 99.97% rating given HEPA filters is based on the efficiency with which they retain 0.3-μm particles (Avery, 1996).

A filtration process can be classified according to the minimum size of particles retained as microfiltration (range 0.1–10.0 μm), ultrafiltration (range 1000–1,000,000 molecular weight), or reverse osmosis (low-molecular-weight molecules, including salts). Microfiltration of water retains bacteria, fungi, and their spores, but it cannot be relied upon to exclude viruses (Block, 1991). Removal of virus from water can be achieved, however, by ultrafiltration or reverse osmosis. Although there are no reports implicating water as a source of adventitious viral infections for laboratory rodents, the possibility should be taken seriously because rodents are susceptible to infection with viruses that are taxonomically related to waterborne human viruses. Characteristically, waterborne viruses are of small to medium size, nonenveloped (and hence stable), and shed in the feces (Block and Schwartzbrod, 1989).

Chemical Disinfection Chemical disinfectants are commonly utilized to decontaminate a room or an isolator before the introduction of SPF animals and to treat the surfaces of materials and containers being brought into an SPF colony or removed from a quarantined colony (Small and New, 1981). Water is often disinfected through chemical processes such as chlorination (Hermann *et al.*, 1982; Homberger *et al.*, 1993) or ozonation (Flood, 1995; Shek *et al.*, 1991). Chemical disinfectants inactivate microorganisms by acting as denaturants that disrupt protein or lipid structures, reactants that form or break covalent bonds, or oxidants (Table 11.2) (Prince *et al.*, 1991). Of these, oxidants such as chlorine dioxide, bleach, vapor-phase H_2O_2, and the peroxygen Virkon® S (from Dupont) are most frequently utilized because they are generally considered more effective than reactants and denaturants for inactivating resistant pathogens such as spore-forming bacteria, nonenveloped viruses and free-living forms of parasites.

The principles of effective chemical disinfection are as follows: (1) starting with a clean surface and freshly prepared disinfectant; (2) applying multiple, or 'layering', chemicals when disinfection requirements are especially strict; (3) allowing adequate contact time as recommended by the disinfectant manufacturer; (4) rinsing if the disinfectant is corrosive to the surface; and (5) selecting disinfectant (s) shown to inactivate the most stable pathogens on your SPF exclusions lists. Various schemes have been developed to link the physicochemical characteristics of microorganisms with susceptibility to chemical inactivation. For example, the Klein–DeForest scheme for viruses, associates sensitivity to disinfectants with viral solubility (Table 11.3). Phenolics and quaternary ammonium compounds, which disrupt lipid membranes, are more potent against lipophilic, enveloped viruses than against hydrophilic, nonenveloped viruses. Oxidants attack all organic compounds and thus inactivate hydrophilic as well as lipophilic viruses (Klein and Deforest, 1983; Prince *et al.*, 1991). A disinfection scale for all microbial taxa likely to be encountered in lab animals, derived from one proposed by Prince *et al.* (1991), is presented in Table 11.4. In brief, this scale recapitulates

TABLE 11.2 Chemical Disinfectant Categories

Category	Examples
Denaturants	Quaternary ammonium compounds (benzalkonium chloride) Phenolics Alcohols
Reactants	Aldehydes (formaldehyde, glutaraldehyde) Ethylene oxide
Oxidants	Halogens (chlorine bleach, chlorine dioxide, povidone-iodine) Peroxygens (vapor-phase H_2O_2, Virkon® S[a])

[a]*Virkon® is a registered trademark of DuPont.*

TABLE 11.3 Klein–DeForest Scheme for Viral Sensitivity to Disinfectants

Category	Solubility	Structure	Sensitivity	Examples
A	Lipophilic	Lipid envelope + capsid	Marked	Paramyxo (Sendai, PVM) Corona (MHV, SDAV) Arena (LCMV)
B	Hydrophilic	Naked capsid	Slight	Picorna (TMEV) Parvo (MVM, MPV, KRV, RPV)
C	Intermediate	Partially lipophilic capsid	Moderate	Adeno (MAV-1,2) Reo (Reo-3) Rota (EDIM, IDIR)

PVM, pneumonia virus of mice; MHV, mouse hepatitis virus; SDAV, sialodacryoadenitis virus; LCMV, lymphocytic choriomeningitis virus; TMEV, Theiler's mouse encephalomyelitis virus; MVM, minute virus of mice; MPV, mouse parvovirus; KRV, Kilham's rat virus; RPV, rat parvovirus; MAV, mouse adenovirus; EDIM, epizootic diarrhea of infant mouse virus; IDIR, infectious diarrhea of infant rat virus.

TABLE 11.4 Approximate Scale for Susceptibility of Laboratory Animal Pathogens to Disinfectants

Susceptibility category[a]	Type of microorganism
A	Enveloped viruses, non-spore-forming bacteria
B	Partially lipophilic, nonenveloped viruses
C	Hydrophilic, nonenveloped viruses
D	Bacterial endospores and parasite ova and cysts

[a]*Susceptibility decreases from A→D.*

the generalization made at the beginning of this section that enveloped viruses and vegetative bacteria are considerably easier to inactivate than are nonenveloped viruses, bacterial endospores, and free-living parasite stages. For the most part, a disinfectant that has been shown to inactivate microorganisms of a particular susceptibility group will inactivate infectious agents in more susceptible groups. For instance, a disinfectant that inactivates parvoviruses (Table 11.3, Category C) will certainly kill non-spore-forming bacteria such as *S. aureus* (Table 11.3, Category A).

The potency of a disinfectant can be enhanced through chemical modification or the addition of synergistic ingredients to the formulation. Conversely, physical factors, including temperature, pH, and the chemical 'demand' of the medium being treated, can diminish potency by reducing the concentration or stability of the active form of the disinfectant. Using chlorine as a case in point, increasing the pH or temperature of water reduces the concentration of hypochlorous acid in favor of the hypochlorite (OCl^-) ion, which is less biocidal. Chlorine is a strong oxidant that reacts not only with living microorganisms but also with inorganic reducing substances such as ferrous iron and organic impurities, including dissolved proteins. These reactions exert a chemical demand that reduces the concentration of free chlorine available for disinfection (Dychadala, 1991; Russell, 1992; Wickramanayake and Sproul, 1991).

Association with dirt and organic matter has been shown to protect microorganisms from disinfectants (Grossgebauer *et al.*, 1975; Russell, 1992; Wickramanayake and Sproul, 1991). Upon colonizing surfaces, bacteria such as *P. aeruginosa* are notorious for forming biofilms, i.e., large clumps of bacteria surrounded in slime, that resist chemical disinfectants (Potera, 1996). It is therefore crucial that soiled surfaces be cleaned before being disinfected in order to reduce chemical demand and to ensure that microorganisms are adequately exposed to disinfectant. Biofilms in water systems can reportedly be removed by treatment with H_2O_2 or alkaline peroxide (Klein and Deforest, 1983; Kramer, 1992).

c. Vectors

Previously in this chapter, it was noted that although biological vectors are rarely involved in the transmission of rodent pathogens, both insects and people have been incriminated as mechanical vectors. People are also carriers of opportunistic bacterial pathogens such as β-hemolytic streptococci and *S. aureus* (Foster, 1996; Patterson, 1996). The keys to controlling insects – mostly flies and cockroaches – are deterrence to entry, sanitation, and the application of control methods, resorting lastly to the use of insecticides that might alter rodent physiology (Small, 1983). Entomologists with a detailed understanding of insect life cycles can often minimize or obviate chemical use. Risk factors for personnel becoming vehicles of infection include (1) exposure to a reservoir, such as an infected colony; (2) access to multiple colonies, especially going from conventional to SPF; and (3) unprotected human–animal contact, as exemplified by a technician handling animals without wearing gloved ideally disinfected or changed between animal groups.

To state the obvious, because people who care for and use research animals do not themselves live in isolators or barrier rooms, contact between people and reservoirs of infection can never be completely avoided. However, practices can be instituted that reduce this risk. Animal care technicians should be prohibited from having pet rodents although enforcement is challenging. There is also a risk associated with procuring rodents from pet stores for feeding snakes or other reptiles kept as pets. In many institutions, visitors are permitted to enter animal facilities only if they have not had recent contact with lab animals. Breeders with large production rooms may have a dedicated staff for each room. Access to smaller colonies, for which a dedicated staff is not practical, should still be restricted, and the flow of people and supplies should always be from 'clean' to 'dirty' areas. Personnel entering a barrier room should gown in a manner that keeps areas of exposed skin to a minimum in order to reduce the potential for transmitting infectious agents. Alternatively, it has become common practice to limit animal–human contact by housing rodents in microisolation cages (Lipman, 1999) or isolators (Trexler, 1983). Contact is limited further by manipulating rodents in a laminar flow hood and by handling them with disinfected gloves or forceps.

d. Cell Lines and Other Biologicals

Inoculation of rodents and other lab animals with untested biologicals, particularly transplantable tumor lines and reagents derived from animal tissues and fluids (Collins and Parker, 1972; Dick *et al.*, 1996; Lipman *et al.*, 2000; Nicklas *et al.*, 1993), has represented a major risk for adventitious viral infections. In fact, many indigenous rodent viruses were discovered as contaminants of animal-derived biologicals that confounded research findings (Bonnard *et al.*, 1976; Hartley and Rowe, 1960; Mckisic *et al.*, 1993; Riley *et al.*, 1960; Rowe and Capps, 1961). Failing to screen biological materials for rodent viruses has also been a public health concern as LCMV has been a relatively prevalent contaminant of cell lines (Bhatt *et al.*, 1986a; Lewis *et al.*, 1965; Simon *et al.*, 1982) and hantaviruses have been isolated from transplantable rat tumors (Lloyd and Jones, 1986; Yamanishi *et al.*, 1983). Traditionally, biologicals were screened for rodent viruses by the mouse and rat antibody production (MAP and RAP) tests (Collins and Parker, 1972; Desousa and Smith, 1989; Lewis and Clayton, 1971; Nicklas *et al.*,

1993; Shek, 1983) as well as other *in vivo* and cultural isolation techniques (Lussier, 1991; Smith, 1986a). Briefly, in an antibody production test, SPF rodents are inoculated with the test article orally and parenterally, held in isolation for a month, and tested by serology for antibodies to rodent viruses; detection of viral antibodies is tantamount to demonstrating infective virus in the test article. MAP and RAP tests continue to be required for cell substrates used in the production of regulated biopharmaceuticals. However, viral PCR tests have replaced antibody production tests of research biologicals because PCR assays are faster, more sensitive, and less costly, and achieve the goal of reduced animal usage espoused in the Three Rs [replacement, reduction, refinement] (3Rs) (Bauer *et al*., 2004; Blank *et al*., 2004).

In addition to being tested for viruses, it is of paramount importance that research biologicals for parenteral injection and those used to produce reagents for animal inoculation are cultured for extraneous bacteria and fungi to demonstrate that they are sterile or at least have a low bioburden. Maintaining a low bioburden is especially challenging for transplantable tumors passaged in animals; moreover, microbial contaminants can include prevalent pathogens such as *H. hepaticus* (Goto *et al*., 2001). A high bioburden is problematic even when free of pathogens because commensal bacteria are more likely to cause disease by circumventing natural host defenses when parenterally injected along with tumor cells into immunodeficient recipients such as nude or SCID mice.

A common contaminant of cells propagated in culture is mycoplasma. Testing cell cultures for mycoplasma infection (usually by culture or PCR) is worthwhile because of the wide range of adverse effects these infections cause such as inhibition of cell growth due to competition for nutrients, cytopathic effects, mutagenesis and interference with viral synthesis, and interferon induction (Hendershot and Levitt, 1985; Mcgarrity *et al*., 1984). However, 99% of the mycoplasma species found in cell culture are of human, porcine, and bovine origin (Erickson *et al*., 1989; Mcgarrity *et al*., 1983; Moore, 1992; Thornton, 1986) and do not infect rodents or rabbits to our knowledge.

A theme of this chapter has been that the complementary approaches of strict control of production processes and comprehensive quality testing, emphasized in CGMPs, are central to microbiological QC of research animals. As neither barrier systems nor the people who maintain rodent colonies are infallible and assays inevitably yield some level of inaccurate results, the complementary approaches of rigorous biosecurity and routine HM are essential to maintaining SPF lab animals. Similarly, QC for biological research reagents should complement testing with procedures to reduce the risk of microbial contamination including (1) obtaining biological reagents from reputable suppliers able to provide material traceability and lot analysis information, i.e., a Certificate of Analysis. Tissues, cells, blood, and so on. should be from animal populations shown to be healthy and SPF according to observations and testing carried out over an extended period. (2) Applying physical or chemical treatments to reagents that are able to withstand them to remove or inactivate infectious agents, e.g., heat inactivation or detergent treatment of serum. (3) Preventing operator-induced contaminations by using PPE, a biological safety hood, and sterile technique. (4) Banking (i.e., cryopreserving vials of) cell lines and microorganisms to ensure that you always have access to starting material that is well characterized and free of extraneous or pathogenic microorganisms, and for which you have documented key information such as designation, lot, species and strain, provenance, preparation method, and QC test results (Shek, 2007).

IV. HEALTH MONITORING

Laboratory testing commonly referred to as 'health monitoring' (HM) is an essential component of a lab animal microbiological QC program. Although the familiar term HM has been used throughout this chapter, specific-pathogen, or microbiological, monitoring (or surveillance) would have been more correct as all but profoundly immunodeficient and disease susceptible research animal strains remain healthy following infections with the highly host-adapted pathogens that are common today. Thus, the main purpose of HM is to detect silent infections of animals and biologicals that nevertheless are capable of confounding research and, if zoonotic, endangering the health of personnel, and to detect those infections early to limit their spread (Lipman and Homberger, 2003).

The traditional HM diagnostic methodologies employed for over half a century are as follows: (1) direct gross and microscopic examinations of animal specimens for pathology, specifically lesions consistent with infectious etiologies, and for parasites; (2) microbiology consisting of cultural isolation of bacteria and fungi and identification of isolates according to their phenotypic characteristics, such as colonial and cellular morphology and biochemical pattern; and (3) serology, i.e., immunoassays of blood or serum samples for antibodies to viruses and several fastidious and invasive microbial pathogens. The newest HM methodology, molecular diagnostics, utilizes molecular genetic techniques to detect and characterize pathogens of all types including viruses, bacteria, fungi, and parasites (Compton and Riley, 2001; Lipman and Homberger, 2003; Livingston and Riley, 2003; Shek and Gaertner, 2002; Weisbroth *et al*., 1998).

The molecular assay method employed most often in HM is the PCR, in which a targeted microbial genomic DNA sequence is specifically and exponentially amplified (i.e., copied) entirely *in vitro* in a matter of hours. Because of exponential amplification, PCR assays characteristically achieve sensitivity levels that far and away surpass those of other test methods. The role of PCR testing in HM has expanded substantially in recent years because PCR assays have been made available for most or all reportable research animal pathogens, including bacteria, fungi, and parasites as well as viruses; moreover, PCR can specifically detect tiny quantities of targeted microbial genomic sequences in a broad array of complex and heavily pooled specimens, including environmental and *ante mortem* animal specimens that are not likely to contain enough viable bacteria and fungi or intact parasites to be suitable for traditional microbiology or parasitology, respectively.

The resident populations and the imported animal groups in quarantine that are being monitored for adventitious infections are referred to as the 'principal' animals. Resident groups comprise the breeding colony and study animals maintained at a facility. HM of resident animal populations verifies the effectiveness of biosecurity measures to eliminate, exclude, or contain and eradicate infections. Animals imported from unapproved sources, such as other research facilities (as opposed to approved commercial breeding colonies) are placed in quarantine; HM determines whether imported animals meet the institutional SPF standards for release from quarantine.

HM has been referred to as direct when it is performed on specimens from the principals and indirect when testing samples from sentinels (Koszdin and Digiacomo, 2002). Direct HM is common for commercial breeding colonies, but otherwise most surveillance has been indirect because lethal sampling of investigator animals for *postmortem* examinations and microbiologic specimen collections is seldom permitted, and in most situations would be impractical and cost-prohibitive. In addition, the principals might be immunodeficient and hence not suitable for serosurveillance.

Irrespective of the diagnostic methodology, detection of a contamination by indirect sentinel surveillance requires transmission of infection from the principals to the sentinels; this occurs most reliably by contact and by exposing sentinels to a high infectious dose of the adventitious agent (Grove *et al.*, 2012; Henderson *et al.*, 2013; Smith *et al.*, 2007). While contact sentinels are an option for imported rodents in quarantine, they are generally inappropriate for routine surveillance of resident animals in popular microisolation caging systems because to be effective the sentinels would have to be moved among resident cages, which, besides being unworkable, would defeat the cage-level barrier. Consequently, sentinels are kept in separate cages supplied with regular changes of soiled bedding pooled from resident cages. Reliance on soiled bedding transmission alone, however, is problematic because microisolation cages impede the spread of infection (Compton *et al.*, 2012; Jensen *et al.*, 2013; Whary *et al.*, 2000a). Thus, the percentage of cages containing contagious animals following an outbreak often remains low and can be as low as 2% (Smith, 2010). The lower the prevalence of infection, the greater the risk that the dose of pathogen in pooled bedding will not be sufficient to infect sentinels. Other factors contributing to this risk include the high degree of pooling that is common because it is typical for a rack of more than 50 cages to have just one or two sentinel cages. In addition, certain pathogens, such as respiratory viruses, host-adapted bacteria, and parasites, are transmitted inefficiently or not at all in soiled bedding (Artwohl *et al.*, 1994; Compton *et al.*, 2004c; Cundiff *et al.*, 1995; Dillehay *et al.*, 1990; Henderson *et al.*, 2013; Ike *et al.*, 2007; Lindstrom *et al.*, 2011; Thigpen *et al.*, 1989). Finally, sentinels can be resistant to infection with certain pathogens due to their age (Riepenhoff-Talty *et al.*, 1985) or genetic background (Besselsen *et al.*, 2000; Filipovska-Naumovska *et al.*, 2010b; Henderson *et al.*, 2015; Hirai *et al.*, 2010; Shek *et al.*, 2005; Thomas *et al.*, 2007).

As described earlier in this section, the exquisite sensitivity and high analytical specificity of PCR assays allow them to detect low concentrations of the targeted microbial genomic sequences in complex, heavily pooled and therefore highly representative specimens not suitable for traditional methodologies. These include specimens from the environment, such as swabs of cages and room or individually ventilated cage (IVC) rack, exhaust air dust, and those that can be collected *ante mortem* directly from animals in residence or quarantine, such as feces and swabs of the upper respiratory tract, skin, and fur (Bauer and Riley, 2006; Dole *et al.*, 2011; Henderson *et al.*, 2013; Jensen *et al.*, 2013). PCR surveillance of these nonsentinel specimens is increasingly employed to lessen the risk of missing adventitious agents that is associated with dependence on transmission of infections to sentinels, particularly those exposed by soiled bedding transfer alone. By eliminating the time required for sentinels to get infected and seroconvert, direct PCR HM of imported animals has been able to reduce the time they spend in quarantine from 2 months or longer for sentinel HM to just 2 weeks. In addition, environmental and direct PCR HM can reduce sentinel usage in accordance with the goals of the 3Rs as well as the expense, logistical, and animal welfare issues of shipping live animals to diagnostic laboratories for pathology and traditional parasitology and microbiology.

A. Methodologies

1. Direct Gross and Microscopic Examination of Animal Specimens

Direct examination continues to be a fundamental diagnostic methodology for pathology and parasitology, despite the increasing availability of rapid and specific *in vitro* PCR and serologic assays for pathogens on SPF exclusion lists. As still common pathogens are highly host-adapted and rarely cause disease, direct examinations should be given a high priority when investigating disease outbreaks. This is highlighted by the pivotal contribution made by gross and microscopic pathology to (1) the discovery of hitherto unrecognized pathogens such as *H. hepaticus*, shown to be the agent responsible for hepatitis and hepatocellular carcinoma in mice in a long-term toxicology study (Fox *et al.*, 1994, 1996; Ward *et al.*, 1994b, 1996), and MNV, found to cause of lethal, systemic disease in mice genetically engineered to be deficient in innate and acquired immunity (Henderson, 2008; Hsu *et al.*, 2006; Karst *et al.*, 2003) and (2) the association recognized pathogens with atypical, novel disease manifestations (Compton *et al.*, 2003; Henderson *et al.*, 2012; Livingston *et al.*, 2011). In addition, direct examination has been a necessary or useful approach to monitor for infectious diseases (Albers *et al.*, 2009; Cundiff *et al.*, 1992; Gibson *et al.*, 1987) and parasite infestations (Watson, 2008) when specific assays have not been available. Finally, direct examination can be used in combination with other test methods to arrive at a specific diagnosis (see below), or to corroborate findings obtained by other assays. For example, microscopic examination of Warthin–Starry silver-stained tissue sections for intracellular bacteria from rodents following disease provocation by immunosuppressive treatment with cyclophosphamide or dexamethasone has been used to verify a preliminary diagnosis of *C. piliforme* infection made by serology or PCR (Nakayama *et al.*, 1984; Riley *et al.*, 1994; Waggie *et al.*, 1981).

a. Techniques

Pathology Tissues and organs are inspected for gross abnormalities during routine HM. Selected tissue specimens, including those with gross abnormalities, may then be fixed in buffered formalin, embedded in paraffin blocks, sectioned onto slides, stained with hematoxylin and eosin, and then examined microscopically for histopathological changes (Weisbroth *et al.*, 1998). Special stains can be applied to tissue sections to enhance the visibility of certain pathogens (Clifford *et al.*, 1995; Gibson *et al.*, 1987; Hoover *et al.*, 1985; Thompson *et al.*, 1982; Waggie *et al.*, 1983; Ward *et al.*, 1994b). In diagnostic and experimental lab animal microbiology, microbial antigens or nucleic acid in tissue sections can be specifically stained by immunohistochemistry (Allen *et al.*, 1981; Brownstein and Barthold, 1982; Cera *et al.*, 1994; Hall and Ward, 1984; Jacoby *et al.*, 1975; Sundberg *et al.*, 1989) or *in situ* hybridization (Gaertner *et al.*, 1993; Jacoby *et al.*, 1995; Smith *et al.*, 1993a), respectively. However, these specific staining techniques are rarely if ever used in routine HM. Instead, the presence of pathogens in tissues is demonstrated by PCR of nucleic acid extracted usually from tissue homogenates, but also fixed tissue sections (Henderson *et al.*, 2012).

Parasitology Low-power dissecting microscopy is used to inspect the pelage and skin of lab animal carcasses for mites and lice, and the macerated gastrointestinal tract for adult helminths (Flynn, 1973; Parkinson *et al.*, 2011). The latter method has been considered the gold standard for diagnosing helminth infestations (Huerkamp, 1993; West *et al.*, 1992). Microscopic examination of skin scrapings may be necessary to detect mites, such as *Demodex* and *Notoedres*, which burrow into the epidermis (Weisbroth, 1979b; Wescott, 1982). It has been reported that fur mites can be found in a higher percentage of mice by microscopic examination of adhesive tape applied to the dorsal fur than by checking the skin or skin scrapings (Ricart Arbona *et al.*, 2010a; West *et al.*, 1992). Fur 'plucks' taken from multiple sites on the dorsal surface (e.g., between the scapulae, near base of the tail) and microscopically examined in Petri dishes also yield reasonably accurate results (Rice *et al.*, 2013), but all of these are limited by sampling 'error' compared to pelt digestion (Owen, 1972), which samples the entire, but deceased, host. Fur mite eggs can also be observed on perianal tape tests used to detect pinworm eggs if the mite infestation is very heavy. Infections with enteric protozoa are diagnosed by examining wet mounts of mucosal scrapings of the small and large intestines with a phase-contrast microscope, which makes it possible to see unstained microorganisms (Brock, 1970; Weisbroth *et al.*, 1996); however, histologic examination of the gastrointestinal tract is best for detecting *Cryptosporidium* (Wasson, 2007). Phase-contrast microscopy is also used for more precise morphologic identification of adult helminths and mites, and to examine fecal floats or centrifugation concentrates for helminth ova and protozoan cysts, tape applied perianally for *Syphacia* pinworm eggs, and tape applied to the fur for mites (Rice *et al.*, 2013; Weisbroth, 1979b, 1998; Weiss *et al.*, 2012).

b. Limitations

Gross and microscopic lesions are seldom diagnostic. Furthermore, direct examinations are characterized by low analytical sensitivity, that is, the lesions and organisms in stained tissue sections and the intact parasite stages that these tests target must be present at high concentrations to be observed, particularly when specimens are examined microscopically as is almost always done. As the level of magnification increases, there is a commensurate rise in the minimum target concentration

required for detection. As an extreme example, the minimum concentration of virus that can be detected by transmission electron microscopy is 10^5–10^6 particles per milliliter (Miller, 1995). High magnification further limits sensitivity by constraining the amount of sample that is practical to examine.

According to sampling statistics (Anonymous, 1976; Clifford, 2001; Dubin and Zietz, 1991; Selwyn and Shek, 1994), the likelihood of detecting an outbreak is enhanced by increasing the number of animals evaluated, but this also increases the labor of sample collection along with the cost of testing. One way of increasing the sample size, while controlling HM costs, is to test sample pools; however, sample pooling is limited by the low analytical sensitivity of direct examinations. Because principal animals at research institutions are seldom made available to be euthanized for routine HM, the number of animals that can be sampled is further restricted by the need to examine *postmortem* specimens for optimal detection of some types of parasites, such as macerated gastrointestinal tract for adult helminths, intestinal scrapings for protozoan trophozoites, and the pelt for ectoparasites (Parkinson *et al.*, 2011). Therefore, *postmortem* specimen collection and examinations are usually restricted to a small number of sentinels (e.g., one or two sentinels per rack). In addition to limiting sample size, the main shortcoming of sentinel surveillance is the risk that sentinels will not become infected (or infested) with pathogens harbored by the principal animals. This is of greatest concern when monitoring resident rodents housed in microisolation cages using sentinels exposed to pooled soiled bedding because, as discussed, certain pathogens including mites (Grove *et al.*, 2012; Henderson *et al.*, 2013; Lindstrom *et al.*, 2011) are poorly transmitted in bedding and sentinels may be exposed to subinfectious doses of pathogens when the prevalence of infection is low, as commonly occurs in microisolation cages, or when sentinels are resistant to an infection due to their age or genetic background.

2. Microbiology: Cultural Isolation and Identification

This section focuses on testing animal specimens for pathogenic microorganisms. Other routine applications of traditional microbiology not covered here are bioburden and sterility testing to monitor the efficacy of disinfection procedures for facilities, equipment, and supplies (Ednie *et al.*, 2005; Meier *et al.*, 2008; Schondelmeyer *et al.*, 2006; Small, 1983). By demonstrating deficiencies in biosecurity measures, monitoring of disinfection processes can help prevent contaminations.

a. Techniques

Cultural Isolation Animal specimens, artificial cell-free agar and broth media, and incubation conditions are chosen to favor the isolation and cultivation of primary and opportunistic microbial pathogens while limiting the growth of commensal and autochthonous microorganisms (Ganaway, 1976; Orcutt, 1980; Weisbroth, 1979a; Weisbroth *et al.*, 1998). The animal sites most often sampled – the upper respiratory tract and large intestine – possess a complex microbiome that can overgrow cultures and obscure colonies of interest. To lessen this problem, specimens are cultured with selective media that contain additives, such as dyes or antibiotics, to inhibit the growth of certain microorganisms. MacConkey's agar, e.g., contains crystal violet and bile salts that selectively inhibit the growth of gram-positive bacteria, while allowing most gram-negative bacteria to grow (Forbes *et al.*, 1998). Media for the isolation of *Helicobacter* spp. from fecal or intestinal specimens contain a mixture of antibiotics to selectively inhibit the growth of the intestinal microbiome (Fox *et al.*, 1994). Overgrowth can be further reduced by culturing sites that do not possess a normal microbiome to obscure invasive bacteria. Tracheal cultures from *Bordetella bronchiseptica*-infected animals contain few extraneous bacteria, making it easier to view *B. bronchiseptica* colonies (Bemis *et al.*, 2003; Brownstein *et al.*, 1985). *Corynebacterium kutscheri* is most reliably isolated from the submaxillary lymph nodes of infected rats (Brownstein *et al.*, 1985). Enrichment media are used to encourage the growth of particular bacteria, which are at low concentration in a specimen containing many microorganisms. Selenite broth is an enrichment medium that is used to recover salmonella from feces or the intestinal tract (Orcutt, 1980). Media are categorized as differential when they allow colonies to be morphologically differentiated based on metabolic characteristics. On MacConkey's agar, lactose-fermenting bacteria produce pink to red colonies, whereas colonies of non-lactose fermenters remain colorless (Forbes *et al.*, 1998). Cultures are usually incubated aerobically at 35–37°C because the majority of clinically important bacteria are facultative anaerobes that will grow under these conditions, whereas the strict anaerobes that constitute the autochthonous microbiome will not. PCR and/or serology are used instead of culture to screen for fastidious and noncultivable microbial pathogens such as *M. pulmonis* (Davidson *et al.*, 1981; Kraft *et al.*, 1982; Loganbill *et al.*, 2005), *Helicobacter* spp. (Whary *et al.*, 2000b), *C. piliforme* (the etiology of Tyzzer's disease) (Goto and Itoh, 1996; Motzel and Riley, 1991; Pritt *et al.*, 2010), cilia-associated respiratory (CAR) bacillus (Cundiff *et al.*, 1994a; Lukas *et al.*, 1987; Matsushita *et al.*, 1987), and *Pneumocystis* spp. (Henderson *et al.*, 2012; Hong *et al.*, 1995; Livingston *et al.*, 2011).

Phenotypic and Genetic Identification After incubation, isolated colonies on agar media are examined to assess their morphology and number; colonies of interest are characterized further. Cellular morphology,

size, and motility are evaluated by examining a wet mount of an isolate with a phase-contrast microscope or a slide of gram-stained cells with a bright-field microscope. Additional tests are performed to determine the identity of isolates suspected to be pathogens. A metabolic profile is established by performing panels of biochemical tests as individual assays (e.g., catalase) and in automated multitest systems (Carroll and Weinstein, 2007; Macfaddin, 1980).

Serotyping may also be necessary or helpful to determine the identity and clinical significance of an isolate. For *Salmonella*, serotypes are based on the somatic O and flagellar H antigens (Ganaway, 1982; Giannella, 1996). β-Hemolytic streptococci usually have group-specific, cell-wall carbohydrate (C) antigens, which are the basis of the Lancefield classification system (Corning *et al*., 1991; Patterson, 1996; Washington, 1996).

As the identification of microbial isolates according to their phenotypic properties may be imprecise or simply inaccurate, biochemical and serologic tests are being augmented or supplanted by PCR and gene sequencing methods that provide highly accurate, reproducible classifications of microorganisms (Dole *et al*., 2010, 2013b; Gentsch *et al*., 1992; Tenover, 1998; Tenover *et al*., 1994; Ushijima *et al*., 1992). Related to their characteristically high analytical specificity and sensitivity, PCR assays can be performed directly on microbially complex clinical specimens, bypassing the need for cultural isolation, which is impractical for routine detection of fastidious microbes such as *Helicobacter* spp. (Whary *et al*., 2000b; Whary and Fox, 2006). So-called next-generation sequencing in which millions of DNA fragments from a single sample are sequenced in unison is already being used to characterize the intestinal microbiome (Friswell *et al*., 2010), but currently is far too expensive and complicated for routine identification of microbial pathogens in clinical specimens (Grada and Weinbrecht, 2013).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an alternative to conventional phenotypic tests for identification of bacterial isolates that, although proposed over 30 years ago, have only recently been made commercially available. Identifications by the MALDI-TOF MS are performed on actively growing cultures, or extracts made from them, and are based on unique peptidic spectra primarily of ribosomal and other housekeeping proteins that are expressed at high levels. The peak intensity and position of the spectra are compared to those in a database to establish identifications. The principal advantages of MALDI-TOF MS in comparison with biochemical testing are that it is much more rapid (completed in minutes instead of hours to days) and has a lower cost per identification, although the mass spectrometer instrument is very expensive. MALDI-TOF MS is also highly reliable and accurate since it is based on molecules that are less dependent on growth conditions and not subject to the expression variability seen in phenotypic systems (Seng *et al*., 2009). Because of the aforementioned advantages, MALDI-TOF MS is increasingly employed, in place of traditional phenotypic techniques, for human and veterinary diagnostic microbiology.

b. Limitations

Microbiology is limited to microorganisms that can be cultivated in cell-free media. Serology and PCR assays have been developed to monitor for noncultivable microbial pathogens such as *C. piliforme*, CAR bacillus, and *Pneumocystis* spp., and have replaced culture when testing for fastidious, slow growing bacteria such as *M. pulmonis* and *Helicobacter* spp. Because the preferred respiratory and intestinal specimens for culture are collected *postmortem*, microbiology of resident animals at research institutions is largely restricted to indirect surveillance of a few sentinels, with the associated risk of missing adventitious infections when the prevalence is low; the pathogen is poorly transmitted in soiled bedding or the sentinels are resistant to infection due to their genetic background or age. The complex microbiome of respiratory and gastrointestinal specimens necessitates that bacteria of interest be viable and present in high numbers in order to obtain isolated colonies for identification.

3. *Serology*

Serology has been the most commonly used methodology to detect rodent infections because it is easily used to monitor viral infections, which are among the most common adventitious agents infecting rodents (Lussier and Descoteaux, 1986; Pritchett-Corning *et al*., 2009; Schoondermark-Van De Ven *et al*., 2006; Zenner and Regnault, 2000). They also have high impact due to their effects on research (Bhatt *et al*., 1986b) and because, unlike parasites, they cannot be treated. Although virus isolation has been used for diagnosis on occasion, that approach can be problematic because (1) many field strains are either difficult to cultivate or noncultivable; (2) virus isolation is time-consuming and expensive (Schmidt, 1979); and (3) live virus may be present in host tissue and shed for relatively short periods of time. By contrast, serum antibody responses are usually detectable by 1–2 weeks post infection and last for long periods (at least months and sometimes for the life of the rodent) and the tests are highly accurate, fast, and relatively inexpensive (Barthold and Smith, 1983; Bhatt and Jacoby, 1985; Homberger *et al*., 1992; Parker and Reynolds, 1968; Peters and Collins, 1981; Smith *et al*., 1984).

a. Techniques

As a consequence of serology's central role in HM, substantial resources have been dedicated over the years toward upgrading the immunoassay and related

technologies it employs. In the mid-1980s, traditional homogenous serologic techniques (with the term homogeneous indicating that sample and assay reagents are mixed and incubated together in solution) such as hemagglutination inhibition (HAI), complement fixation, and virus neutralization were supplanted by more sensitive and broadly applicable heterogeneous solid-phase immunoassays, notably the indirect enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) schematically depicted in Figs. 11.2 and 11.3b,

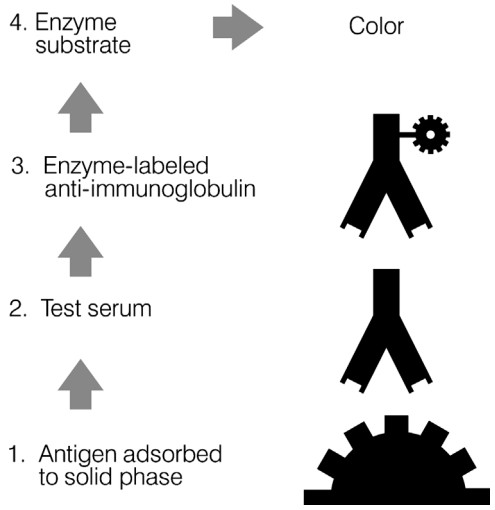

FIGURE 11.2 Indirect ELISA for microbial antibodies. Antigen, such as virus particles or lysates, microbial cell extracts, or recombinant proteins, is adsorbed to wells in microtiter plates. Separate wells may be coated with a 'tissue control', i.e., an extract of uninfected host cells (or of a related microorganism) to detect nonspecific binding of Ig. The numbers denote incubations; incubations 1–3 are followed by washing to remove unbound antibodies and other substances that might interfere with the assay. The rate at which chromogenic substrate is converted to a colored product is proportional to the amount of enzyme-labeled anti-Ig and, hence, serum antibodies bound to the solid phase. The color development can be scored visually, but is more commonly measured as optical density by an ELISA plate reader. *Adapted from Mahony and Chernesky (1999), Fig. 4A, p. 208.*

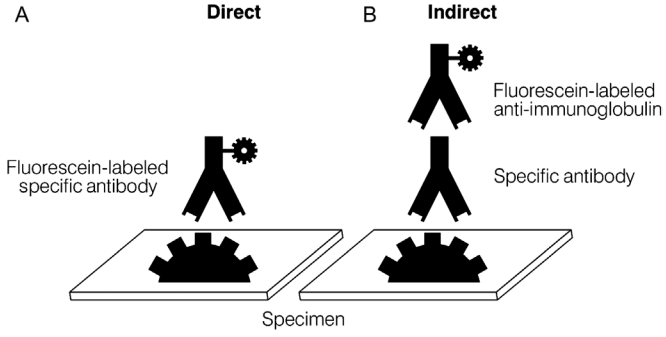

FIGURE 11.3 Direct (A) and indirect (B) immunofluorescence assays. *Adapted from Mahony and Chernesky (1999), Fig. 2, p. 206.*

respectively (Kraft *et al.*, 1982; Mahoney and Chernesky, 1999; Parker *et al.*, 1979; Smith, 1983a, b, 1986b; Takahashi *et al.*, 1986; Voller *et al.*, 1982). The 'solid phase' denotes the surface to which the antigen is attached. For ELISA, antigen, consisting of purified virus particles, microbial cell extracts, or recombinant proteins encoded by microbial genes of interest, is immobilized on the surface of wells in microtiter plates made of specially prepared polystyrene or polyvinyl; separate wells may be coated with an extract of uninfected host cells (or with antigen from other microorganisms), often called a 'tissue control', to detect nonspecific binding of immunoglobulin (Ig). For IFA, infected and uninfected cells are fixed to wells on glass slides. The fixative is usually cold acetone, which permeabilizes the cell membrane, making the intracellular viral antigens accessible to antibodies in the serum samples. 'Heterogeneous' indicates that each incubation period is followed by a wash step to separate antibody bound to the solid phase from unbound antibody. The wash step also removes interfering substances in a specimen that could compromise the sensitivity or specificity of a corresponding traditional homogeneous test. 'Indirect' refers to detection of serum antibodies bound to the solid phase (whether as specific antigen–antibody complexes or nonspecifically) by labeled anti-immunoglobulin (anti-Ig), such as goat IgG anti-mouse IgG, or Ig-binding bacterial proteins including Proteins A and G (Delellis, 1981; Hrapchak, 1980). Common non-radioisotopic labels include the enzymes horseradish peroxidase and alkaline phosphatase for ELISA and the fluorescent dye fluorescein for IFA (Mahoney and Chernesky, 1999; Voller *et al.*, 1982).

A direct solid-phase immunoassay, as shown in Fig. 11.3a, is one in which a label is coupled to the microbial antibodies rather than anti-Ig or Ig-binding bacterial protein for indirect labeling. Although labeled microbial antibodies are incorporated into blocking or competitive assays for serum antibodies (Vonderfecht *et al.*, 1985), they are more commonly used in fluorescence and enzyme immunoassays when doing research on the time course and distribution of infections *in situ*, i.e., in animal tissues and cells (Allen *et al.*, 1981; Brownstein and Barthold, 1982; Cera *et al.*, 1994; Dick *et al.*, 1996; Jacoby *et al.*, 1975; Kimsey *et al.*, 1986; Sundberg *et al.*, 1989; Weir *et al.*, 1988) and in specimens using techniques such as the double-antibody sandwich antigen capture method (Jure *et al.*, 1988; Newsome and Coney, 1985; Vonderfecht *et al.*, 1988). While solid-phase antigen capture immunoassays for pathogens have been popular in domestic and companion animal and human diagnostics, they have seldom been utilized in laboratory HM for a variety of reasons. First, as already discussed, the window of active infection and shedding is often short-lived. Even when an infection is active, the concentration of microbial antigens in animal specimens may be below the assay's detection

limit, which is why MPV pathogenesis studies have utilized *in situ* hybridization (Jacoby *et al*., 1995; Smith *et al*., 1993a) and PCR (Besselsen *et al*., 2000, 2007) to detect viral DNA rather than antigens. Also, antigen immunoassay development, particularly obtaining or producing microbial antibodies of the appropriate specificity, can be a long and expensive process with an unpredictable outcome; this helps explain why most of the few evaluations of antigen-capture immunoassays for HM have utilized commercial kits that had been developed for other host species, but recognized a group antigen shared with the related lab animal pathogen. Human group-A rotavirus kits, for instance, have been assessed for detection of mouse rotavirus in fecal specimens (Jure *et al*., 1988; Newsome and Coney, 1985). Finally, the importance of antigen-capture immunoassays in diagnostics has been substantially diminished by the advent of more sensitive and specific molecular genetic assays – especially PCR assays – that are better suited to direct detection of infectious agents in animal and environmental samples (Wilde *et al*., 1990).

After the labeled antibody incubation and final wash (Fig. 11.2), ELISA reactions are developed by adding an enzyme substrate to test plate wells; most substrates are chromogenic, that is, they are converted by the enzyme to a colored product at a rate proportional to the quantity of enzyme-labeled anti-Ig and, hence, serum antibodies attached to the solid phase. Color development can be read visually in a qualitative or semiquantitative fashion, but is usually read with a spectrophotometer, or ELISA plate reader, that exports optical density readings to a computer for analysis and reporting (Mahoney and Chernesky, 1999; Voller *et al*., 1982). By contrast, IFA reactions are examined manually using a fluorescence microscope; analysts classify test serum reactions as negative to strong positive or nonspecific by comparing the pattern, intracellular location, and intensity of fluorescence to those observed with standard immune and nonimmune control sera (Lyerla and Forrester, 1979).

Because ELISA are generally performed in 96-well microtiter plates and reactions can be instrument-read and processed by computer, they are better suited than IFA to high-throughput testing and, hence, have been preferred for primary screening of serum samples. The IFA, however, has proven to be an excellent method for confirmatory testing because the pattern of fluorescence and whether it is located in the host cell nucleus, cytoplasm, or both is useful in distinguishing specific from nonspecific reactions. In addition, IFA are generally as sensitive as corresponding ELISA (Homberger *et al*., 1995; Kraft *et al*., 1982; Smith, 1983a) and they can be more 'inclusive', i.e., better able to detect seroconversion to heterologous viral strains and serotypes (OIE, 2013), when the host develops antibodies to highly conserved nonstructural viral protein antigens found in the infected cells that compose IFA antigen, but not necessarily in ELISA antigen consisting of purified virus particles. The inclusivity of the IFA (specifically, its ability to detect antibodies to the nonstructural proteins conserved among rodent parvoviruses in mouse and rat populations that were largely MVM and rat virus (RV) seronegative by serotype-specific HAI and by ELISA with virus particle antigen) provided the initial evidence for the existence of then-novel rodent parvoviruses later identified as MPV, rat parvovirus, and rat minute virus (Jacoby *et al*., 1996).

The prior rodent parvovirus example demonstrates that inclusivity is a preferred attribute of primary assays because it can enhance diagnostic sensitivity, i.e., the proportion of infected animals that test positive. Moreover, a single inclusive assay can replace several 'exclusive', i.e., strain-specific tests, thereby reducing the number tests and the cost of surveillance; however, this comes with a risk of false-negative (FN) findings if the antibody response to the conserved antigen (variously called shared or group-specific antigens) is delayed, weak, or absent, as has been demonstrated for the antibody response of rodents to the parvovirus nonstructural protein NS1 (Besselsen *et al*., 2000; Filipovska-Naumovska *et al*., 2010a; Henderson *et al*., 2015; Livingston *et al*., 2002). Solid-phase immunoassays tend to be more inclusive than corresponding traditional tests because they can detect antibodies to any of the epitopes (i.e., antigenic sites) presented by the microbial antigens attached to the solid phase and at much lower levels (Parker *et al*., 1979), which improves detection of low-titered 'cross-reacting' antibodies to shared or group-specific antigens. By contrast, virus neutralization and HAI are highly exclusive because by definition they only recognize antibodies to viral surface protein antigens that are unique to the serotype or strain of virus being used in the test. Although this high level of exclusivity is not favored for primary surveillance, it is desirable for confirmatory testing to delineate the strain or serotype specificity and, thus, the etiology of the viral antibody response (Parker *et al*., 1965). The inclusivity and high analytical sensitivity of solid-phase immunoassays has expanded lab animal serosurveillance to pathogens for which traditional serologic tests had not been developed because they were not sufficiently sensitive or applicable. Examples of agents added to serologic panels after the advent of solid-phase methods are mouse rotavirus (Smith) and invasive microbial pathogens including *M. pulmonis* (Cassell *et al*., 1983; Minion *et al*., 1984), *C. piliforme* (Motzel and Riley, 1991; Waggie *et al*., 1987), *Helicobacter* spp. (Fox *et al*., 1996; Whary *et al*., 2000b), and CAR bacillus (Lukas *et al*., 1987; Matsushita *et al*., 1987), *Pneumocystis carinii* (Henderson *et al*., 2012; Hong *et al*., 1995) and *Encephalitozoon cuniculi* (Digiacomo *et al*., 1983).

To be performed optimally, ELISA and other solid-phase immunoassays generally require antigen that is more concentrated and pure than was needed for the traditional homogenous tests they replaced. During the 1990s, advances in recombinant DNA technology made it possible to produce large quantities of recombinant protein rapidly from a cloned gene of interest inserted into microbial, mammalian, or baculovirus (an insect virus) expression vector systems. These systems have facilitated the production of pure and potent antigen, particularly for infectious agents that are fastidious, noncultivable, or zoonotic (Ball-Goodrich *et al.*, 2002; Filipovska-Naumovska *et al.*, 2010a; Homberger *et al.*, 1995; Katz *et al.*, 2012; Schmaljohn *et al.*, 1990). Thus, these systems have obviated the need to propagate a pathogen in culture and the recombinant proteins they generate are noninfectious. In addition, microbial genes of interest have been 'fused' to sequences that encode affinity tags, such as 6 × histidine, to permit purification of the recombinant proteins by affinity chromatography (Ball-Goodrich *et al.*, 2002; Riley *et al.*, 1996b; Seletsakia *et al.*, 2004). Recombinant viral capsid proteins, such as the parvovirus VP2, that self-assemble into virus-like particles, or VLPs, can also be purified by gradient centrifugation and other conventional techniques (Kahn *et al.*, 2008; Livingston *et al.*, 2002). Finally, incorporating recombinant protein antigens representing different viral strains and proteins into serosurveillance panels has enhanced diagnostic accuracy by being more inclusive and permitting confirmation and characterization of antibody specificity.

In the 2000s, novel systems became commercially available for assay multiplexing, i.e., for performing an array of tests simultaneously, in a single well, tube, or chip location. Among the most popular of these for lab animal serology has been Luminex's Multi-Analyte Profile (xMAP) platform (Adams and Myles, 2013; Besselsen *et al.*, 2008a; Hsu *et al.*, 2005; Khan *et al.*, 2005; Wunderlich *et al.*, 2011), which is termed a suspension microarray because the solid phase is a color-coded 5.6-μm polystyrene bead available in at least 100 different color sets. At the conclusion of an assay procedure, beads from test wells flow single file through an array reader (which is a modified flow cytometer); each bead is interrogated by two laser beams: one to identify the bead color set (i.e., test) and the other to measure the intensity of fluorescence emitted by the reporter dye phycoerythrin (Fig. 11.4). A predetermined minimum number of beads are read for each assay (i.e., bead set) in a well and the intensity of fluorescence for each test is reported as median fluorescence intensity (MFI) (De Jager *et al.*, 2003; Richens *et al.*, 2010).

The xMAP antibody immunoassays for serosurveillance, abbreviated as MFI or MFIA for multiplexed fluorescence or fluorometric immunoassay, are indirect,

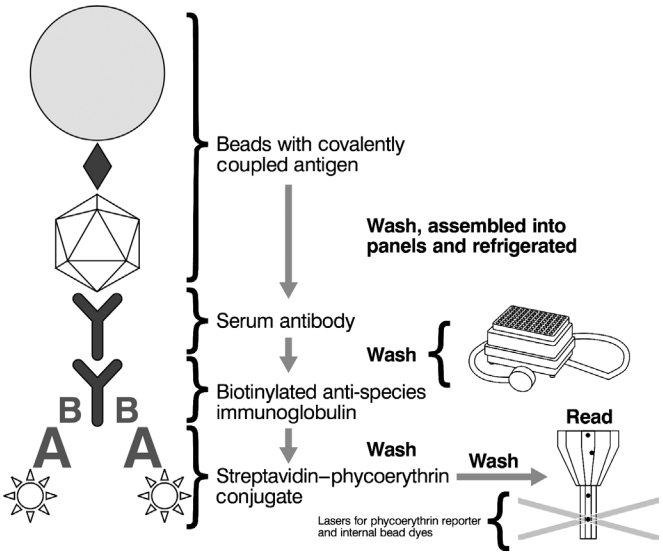

FIGURE 11.4 Multiplexed fluorometric immunoassay. An antigen or control (e.g., tissue control) is covalently coupled to beads of an assigned color set, of which there are 100. Serum antibodies bound to the bead are labeled with the reporter dye phycoerythrin by incubations with biotinylated anti-species Ig (e.g., goat IgG anti-mouse IgG) and phycoerythrin-conjugated streptavidin. An array reader evaluates a predetermined minimum number of beads of each color set in the panel. Each bead is interrogated by two laser beams, one to identify the bead color set, which corresponds to a test, and the other to measure the intensity of fluorescence emitted by the reporter dye phycoerythrin. The array reader reports the phycoerythrin median fluorescence intensity (MFI) for each assay.

heterogeneous solid-phase tests. Hence, the MFIA steps depicted in Fig. 11.4 are essentially the same as those described for the ELISA. Briefly, an antigen or control (e.g., tissue control) is covalently coupled to beads of a specific color. Although up to 100 different tests could be included in an MFIA panel, the largest serologic panels for HM contain fewer than 40. Antigen–antibody complexes that form during the serum incubation (as well as nonspecifically bound antibodies) are detected by phycoerythrin-conjugated anti-Ig or by biotinylated anti-Ig followed by phycoerythrin-conjugated streptavidin (a protein that binds strongly to biotin). The array reader reports the MFI for each assay in a well as described (Wunderlich *et al.*, 2011). Overall, the diagnostic performance of MFIA has been shown to be comparable to that of ELISA (Khan *et al.*, 2005).

Multiplexing has been a very important advance for serosurveillance because an extremely small volume of serum (e.g., 1–2 μl for MFIA) suffices for even the largest surveillance panels. Moreover, multiplexing conserves reagents, reduces the volume of waste fluids, and permits high-throughput testing without complex and expensive automation. These efficiencies have made it practical to add confirmatory antibody assays for common adventitious agents and internal controls to panels

to enhance the quality and reliability of serologic findings. In addition to standard tissue control bead sets for detecting nonspecific binding of serum Ig, MFIA panels have incorporated a bead set coated with Ig of the test species to detect procedural errors such as the failure to add labeled anti-Ig to a well and a set coated with anti-test species Ig to identify samples with inadequate levels of Ig; failures of the latter control occur most often because the sample is from an animal that is immunocompromised or different from the test species.

The dried blood spot (DBS) has recently been shown to be a suitable alternative to serum for serology in large part because multiplexing has made it feasible to perform the largest test panels on the single drop of blood used to prepare a DBS. Although new to HM, DBS technology has long been used for human neonatal screening (Mei *et al*., 2001) and is increasingly employed for serial sampling of rodents in pharmacologic studies (Beaudette and Bateman, 2004). Collecting the drop of blood for preparing a DBS is minimally invasive and therefore can easily be performed on unanesthetized animals. In addition, the use of DBS eliminates the steps, reagents, and equipment required for serum preparation and DBS can be safely shipped in envelopes at ambient temperature in contrast to serum samples. *Ante mortem* blood collection for preparing DBS facilitates direct sampling of colony animals to supplement sentinel monitoring, to verify positive sentinel findings, and to identify infected animals to be culled.

b. Limitations

Despite the rapidity and low cost of serologic tests, they do have some important limitations. They are applicable mainly to viruses and seldom used for bacteria, with the exception of *M. pulmonis*, CAR bacillus, and *E. cuniculi*, or fungi due to poor specificity compared to viruses, which are less complex with their smaller genomes. Bacteria and fungi also induce weak antibody responses unless they are invasive. Poor sensitivity can occur when antigen purified from one bacterial strain does not cross-react with antibodies to others (Manning *et al*., 1994). Additional limitations of serology include the requirement for an immunocompetent host that is susceptible to infection by virtue of age and genotype. Thus, serology is not suitable for direct testing of known immunodeficient or 'immunovague' genetically engineered principals to confirm or eradicate an adventitious infection. There is also a period of at least a week to 10 days between infection and seroconversion.

4. *Molecular Diagnostics – PCR*

The revolutionary advances in molecular genetics that have gained pace in recent decades have caused a shift from the just-described traditional diagnostic methodologies, which identify pathogens by their phenotypic characteristics (e.g., morphology, biochemical profile, serotype, or serum antibody specificity) to molecular assays for specific microbial gene sequences. Key among these advances has been the development and general availability of robust, rapid, and inexpensive tools for (1) amplifying, cloning, and sequencing genes; (2) analyzing and comparing gene sequences to identify those that are shared by related pathogens or strain-specific to target for inclusive surveillance or exclusive confirmatory assays, respectively; and (3) selecting and synthesizing DNA (or RNA) fragments with nucleotide sequences complementary to those targeted. This highly engineered process has led to the very rapid development of extremely sensitive molecular assays for newly recognized as well as known infectious agents of all types, with specificities that are generally more predictable and definitive than those of phenotypic tests (Tang and Persing, 1999; Tenover, 1998).

a. Techniques

The annealing of a known fragment (or collection of fragments) of RNA or DNA to complementary RNA or DNA sequences in a sample is fundamental to the principal molecular assay strategies (for infectious agents) of (1) labeled probe hybridization and (2) biochemical amplification. Reporter probes (usually 100–1000 bases long) for hybridization assays are directly or indirectly labeled with a radioisotope, an enzyme that acts on a chromogenic or chemiluminescent substrate, or a fluorescent dye. Assays begin with immobilization of the sample nucleic acid *in situ* or by blotting onto nitrocellulose or nylon membranes. For example, Southern (after the developer E.M. Southern) and Northern (a play on words) refer to blots of DNA and RNA, respectively (Cundiff *et al*., 1994b; Hsu and Choppin, 1984). Alternatively, target sequences in a specimen can be captured by an unlabeled probe attached to a solid phase, e.g., a chip, bead, or microtiter plate well (Goto and Itoh, 1996). Prior to hybridization, double-stranded sample and probe DNA must be denatured to single-stranded DNA by heating (e.g., 90–100°C) or exposure to alkaline conditions. The reaction mixture is then cooled (to 55–65°C) to permit the formation of stable probe-target hybrids (which can be RNA to RNA, DNA to DNA, or DNA to RNA). Raising the temperature of incubation during hybridization enhances assay specificity by increasing the degree of complementarity necessary for stable probe–target hybrids to form. Free probe can be removed by washing (as is done in heterogeneous solid-phase immunoassays) or digestion with an enzyme that attacks single-stranded nucleic acids. Finally, the degree of hybridization is determined by measuring the signal emitted by the probe label or the enzyme-substrate product (Fig. 11.5) (Tang and Persing, 1999; Tenover, 1998).

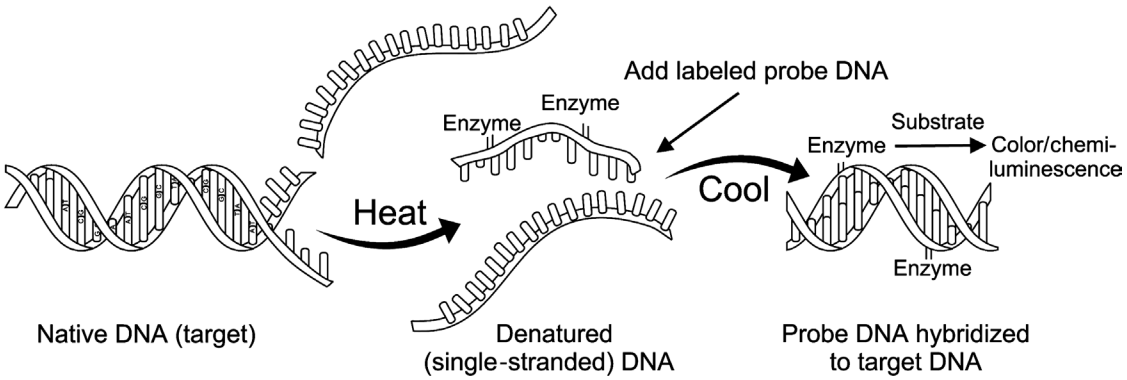

FIGURE 11.5 Hybridization with an enzyme-labeled DNA probe. Native (double-stranded) DNA is denatured by heating (to 90–100°C). The reaction mixture is then cooled (to 55–65°C) to permit the formation of stable probe–target hybrids. Free probe is removed by washing or by digestion with an enzyme that attacks single-stranded nucleic acids. The degree of hybridization corresponds to the amount of color or luminescence from the enzyme-substrate product. *Adapted from Tenover (1998), Fig. 14-1, p. 153.*

The sensitivity of probe hybridization assays for direct detection of pathogens in clinical specimens, like that of immunoassays, is constrained by the quantity of organisms typically found in specimens and background due to nonspecific binding of the labeled probe (Mahoney and Chernesky, 1999; Tang and Persing, 1999). These limitations have been overcome by the development of practical and robust technologies for rapid biochemical amplification, or copying, of target (or probe) nucleic acid sequences entirely *in vitro*. The best developed and most widely used of these, the PCR, was the invention for which Kary Mullis was awarded the Nobel Prize for Medicine in 1993 (Mullis, 1990). Because of its versatility, speed, exquisite sensitivity, and definitive specificity, PCR has become the preeminent diagnostic technique for demonstrating infectious agents (including those of lab animals) in clinical and environmental specimens and biologics.

The PCR consists of repeated cycles of heating and cooling, termed thermal cycling, during which a DNA template is enzymatically replicated, i.e., amplified (Fig. 11.6). The repeated, sequence-specific amplification that occurs in PCR is enabled by (1) synthetic oligonucleotide primers (15–25 bases long) that anneal in opposite directions to complementary strands of the DNA template at sites separated by up to 500 base pairs (for surveillance assays) and (2) a heat-stable DNA polymerase (such as the Taq DNA polymerase originally isolated from the thermophilic bacterium *Thermus aquaticus*) with the unique ability to tolerate the 95°C denaturation step in a PCR cycle (Cooper, 1997; Tang and Persing, 1999; Tenover, 1998).

Primers are designed by analyzing sequence data obtained from published sources (e.g., databases and scientific journals), from colleagues, and by DNA sequencing of laboratory and field strains of the agent of interest (Ball-Goodrich and Johnson, 1994; Battles *et al.*, 1995; Besselsen *et al.*, 2006; Henderson *et al.*, 2012). To reduce the risk of missing an adventitious infection, primary PCR assays for microbiological surveillance maximize inclusivity by using primers targeting genes that are conserved among strains of a pathogen, such as the nonstructural NS-1 gene of parvoviruses (Besselsen *et al.*, 1995a), or a mixture of primers that account for strain variation. By contrast, the secondary PCR assays for confirming or investigating outbreaks frequently emphasize exclusivity by targeting genes that differentiate among pathogen variants, such as the capsid gene of parvoviruses (Besselsen *et al.*, 1995b). PCR primers for bacteria and parasites chiefly target sequences in the ribosomal genes (Battles *et al.*, 1995; Beckwith *et al.*, 1997; Fox *et al.*, 1994; Goto and Itoh, 1996; Greisen *et al.*, 1994; Grove *et al.*, 2012; Loganbill *et al.*, 2005; Shames *et al.*, 1995), which have been extensively analyzed and contain both conserved and differential regions, but sequences in other well-characterized genes are targeted too, for instance, the RNA polymerase *rpoB* gene of bacteria (Dole *et al.*, 2010, 2013b; Gundi *et al.*, 2009).

Although the PCR can only copy DNA, RNA templates such as the genomes of MHV (Casebolt *et al.*, 1997; Homberger *et al.*, 1991; Matthaei *et al.*, 1998) and MNV (Hsu *et al.*, 2006; Taylor and Copley, 1993) can be detected by PCR provided they are first transcribed by a reverse transcriptase to a complementary DNA template. PCR assays of this type are referred to as reverse transcription (RT) PCR (RT-PCR). Priming options for RT include sequence-specific primers, which are the most efficient, and nonspecific primers such as oligo-dT (which anneal to the polyA sequence appended to RNA transcripts) and random hemaxers. There are advantages and disadvantages of each primer type, but nonspecific priming of RT is preferred when testing for multiple RNA viruses (Compton and Riley, 2001; Henderson *et al.*, 2013; Lifetechnologies, 2014).

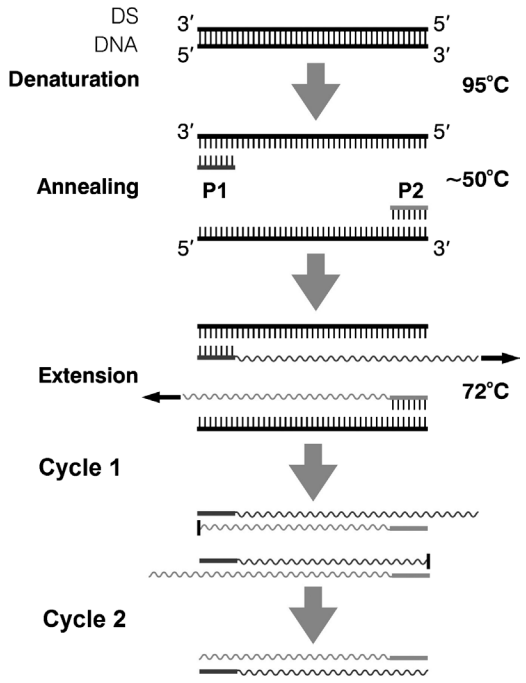

FIGURE 11.6 Steps of PCR. First, nucleic acid isolated from a clinical specimen is denatured at high temperature. Next, the reaction temperature is lowered to allow the oligonucleotide primer pair (P1 and P2) to anneal to complementary target microbial sequences. Last, the heat-stable DNA polymerase synthesizes copies of the target sequences by extending the primers. Copies made in a cycle act as template subsequent cycles, resulting in exponential amplification (Fig. 11.7).

As illustrated in Fig. 11.6, each PCR cycle comprises three steps including denaturation, annealing, and elongation. Following denaturation (at approximately 95°C), reactions are cooled to the annealing temperature, which varies (from 50 to 70°C) according to the melting temperature of the primer–template hybrid, and then reheated (e.g., to 72°C) for the elongation step during which the DNA polymerase synthesizes complementary DNA strands by extending the hybridized primer. Elongation of the forward and reverse primers (which match sequences in the 5′ results in complementary sense and antisense strands of DNA, respectively). A PCR assay consists of 30–50 of these cycles, each lasting no more than several minutes and performed automatically by a programmable heating block called a thermocycler. As the PCR progresses, DNA synthesized in one cycle serves as a template for subsequent cycles, setting in motion a chain reaction (hence, the name polymerase chain reaction) in which the targeted gene sequence is exponentially amplified (Fig. 11.7). For example, after 25 cycles, the PCR can theoretically produce 100,000 copies from a single starting copy of the gene of interest.

In the standard 'gel-based' method, the PCR product, or amplicon, is identified in an ethidium bromide-stained gel electrophoretogram exposed to UV light as a visible fluorescent band of an expected size. To rule out nonspecific amplification, the identity of a PCR product determined by size can be substantiated by sequence-specific methods such as restriction enzyme analysis, DNA sequencing, or labeled probe hybridization (Besselsen *et al.*, 2006; Goto and Itoh, 1996; Goto *et al.*,

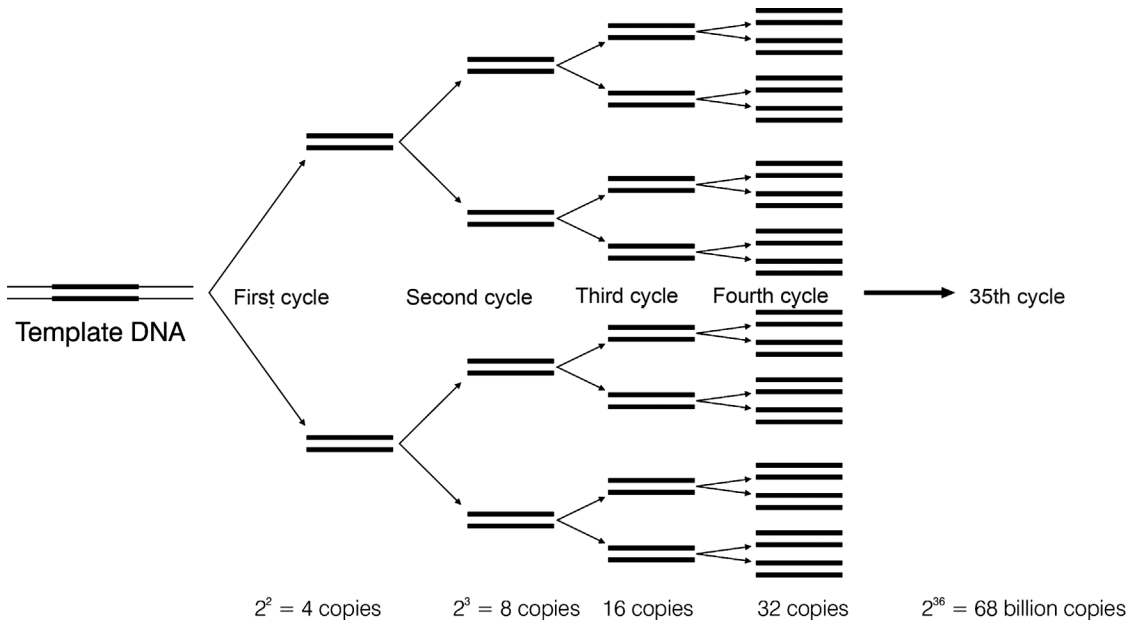

FIGURE 11.7 Exponential amplification by PCR. DNA synthesized in one cycle serves as template for subsequent cycles, setting in motion a chain reaction in which the targeted gene sequence is exponentially amplified.

1998; Riley *et al.*, 1996a; Xiao *et al.*, 1992). The latter is increasingly used as the primary method for identifying PCR product because it is more specific, sensitive, and amenable to automation and computer data processing than the gel-based method (Tang and Persing, 1999).

The most widely used PCR technique that relies on labeled probe hybridization for sequence-specific identification of amplicons is the fluorogenic nuclease, or TaqMan, assay, in which amplification and hybridization occur concurrently (Heid *et al.*, 1996; Holland *et al.*, 1991). The TaqMan probe is an oligonucleotide that anneals to a DNA template sequence between the forward and reverse primers and is tagged on opposite ends with a fluorescent reporter dye and a quencher dye. As long as the probe is intact and the dyes are in close proximity, the reporter signal is quenched. But when extending a primer, the Taq DNA polymerase uses its 5′–3′ exonuclease activity to digest annealed probe (Fig. 11.8A). The resultant separation of the reporter from its quencher generates a sequence-specific fluorescent signal that can be read after each amplification cycle, i.e., in 'real time', or once at the end of the PCR assay. In the real-time assay, results are reported as the number of cycles required to reach a (low) threshold signal, or Ct (for cycle threshold), which is inversely related to the copies of DNA template added to the reaction (Gibson *et al.*, 1996; Heid *et al.*, 1996; Kendall *et al.*, 2000; Kutyavin *et al.*, 2000; Leutenegger, 2001) (Fig. 11.8B). To avoid the confusion that would arise if both the real-time and reverse transcription PCR used the prefix RT, real-time assays are referred to as quantitative (q) PCR. Particularly for pathogens that are present in very high copy numbers in specimens from infected animals, estimating the copy number is helpful for identifying and discounting low-copy positive results due to contamination with template from other samples, controls, or the environment. Other important advantages of the TaqMan technique in comparison with the gel-based method include better analytical specificity and sensitivity due to the internal probe, less risk of contamination because reaction tubes stay closed post amplification, and higher throughput as there are no post-PCR processing steps and reactions are automatically read by a fluorometer and transferred to a computer for analysis and reporting. Moreover, several systems are available for creating spatial multiplexes of TaqMan PCR, including the OpenArray platform, which has been used in research animal HM (Henderson *et al.*, 2013) and biologics testing. As each test in an OpenArray chip occupies a separate location, the OpenArray avoids the pitfalls of competitive inhibition (Hamilton *et al.*, 2002) and low specificity (Lo *et al.*, 1998) that can affect standard homogenous PCR multiplexes created by mixing of multiple primer sets together in a single well. Because of these advantages, fluorogenic nuclease PCR has been developed for many pathogens of rodents including viruses (Besselsen *et al.*, 2003; Blank *et al.*, 2004; Drazenovich *et al.*, 2002; Ge *et al.*, 2001; Redig and Besselsen, 2001; Uchiyama and Besselsen, 2003; Wagner *et al.*, 2003, 2004), microbes (Dole *et al.*, 2010, 2013b; Drazenovich *et al.*, 2002; Ge *et al.*, 2001; Henderson *et al.*, 2012; Whary *et al.*, 2001), and parasites (Dole *et al.*, 2011; Jensen *et al.*, 2013; Rice *et al.*, 2013; Weiss *et al.*, 2012).

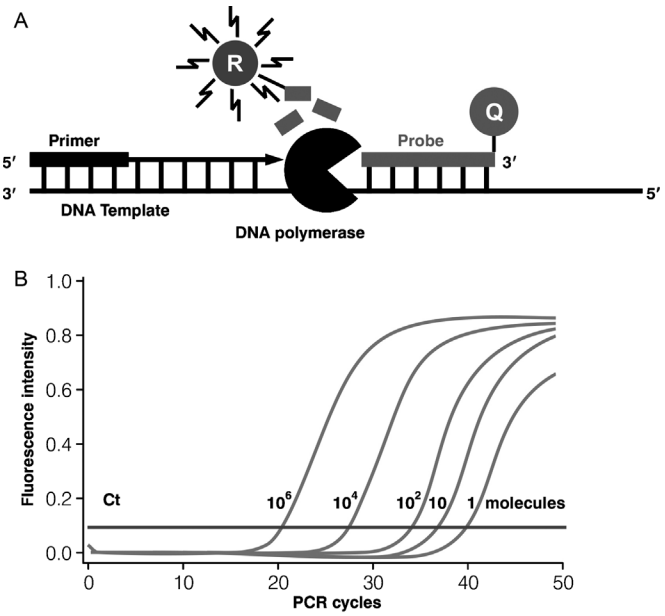

FIGURE 11.8 Real-time fluorogenic nuclease qPCR. The oligonucleotide probe tagged on opposite ends with a fluorescent reporter dye (R) and a quencher dye (Q), anneals to the DNA template between the forward and reverse primers. During the extension phase of the cycle, the DNA polymerase uses its 5′–3′exonuclease activity to digest annealed probe; cleavage of the report dye from the probe (and thus, separation from the quencher) generates a sequence-specific fluorescent signal (A). When the fluorescence intensity is read in 'real time', i.e., after each amplification cycle, the number of cycles required to reach a threshold signal (Ct) is inversely related to the copies of DNA template added to the reaction (B).

The exponential amplification that accounts for the extreme sensitivity of PCR assays is analogous to culture, with the advantages that PCR is completed in hours instead of days to weeks, avoids the biosecurity and health risks associated with propagating pathogens, is applicable to fastidious and noncultivable agents, and is minimally affected by the complexity of the specimen microbiome because amplification is sequence-specific. PCR can detect low concentrations of infectious agent template in heavily pooled and therefore highly representative samples such as room or ventilated rack exhaust air dust and noninvasive specimens collected *ante mortem* directly from animals in residence or quarantine – samples that are unlikely to contain enough viable bacteria and fungi or intact parasites to be suitable for traditional microbiology or parasitology, respectively.

Thus, by allowing testing of principal and environmental samples, PCR addresses the main shortcomings of sentinel HM, which is that it depends on transmission of adventitious infections of the principal animals to the sentinels. This transmission, however, may not occur when the adventitious agent is inactivated in soiled bedding; the percentage of actively infected animals is low as is common for rodents housed in microisolation caging systems; or sentinels are resistant to infection due to their age or genetic background.

b. Limitations

Detection of microbial nucleic acid template by PCR can occur in the absence of infection. The exquisite sensitivity of the PCR makes it especially vulnerable to false-positive (FP) findings following even minute levels of template contamination from test samples (and is most likely to occur for prevalent organisms that are present in high copy numbers), controls, the environment, and so forth. This risk of contamination, which represents a significant challenge to high-throughput screening, can be reduced by physical separation of pre- and postamplification procedures, decontamination of work surfaces with chemicals or UV irradiation, and enzymatic digestion or chemical inactivation of amplified template. Estimating the copy number by qPCR can be helpful in discounting low-copy positive results not associated with an active infection.

PCR may miss infections with viruses and other pathogenic organisms that are shed transiently, particularly in sentinels tested quarterly, although PCR has been shown to detect MHV and MPV in feces for weeks to months after infected mice are no longer contagious (Besselsen *et al.*, 2007; Compton *et al.*, 2004a). Furthermore, PCR continues to detect template in exhaust air dust swabs even after principal and sentinel animals have stopped shedding a pathogen. PCR results can be FN due to sample-mediated inhibition, but this can be detected by including an internal control assay or by spiking a duplicate reaction with template. Finally, PCR is still relatively labor-intensive and costly, although the cost of PCR testing can be contained by pooling of samples from various animal sites appropriate to the organisms being detected and the environment.

B. Factors Affecting Accuracy of HM

The importance of HM results that correctly represent the current pathogen status of the principal populations being monitored has been accentuated by (1) the continued incidence of outbreaks, particularly with environmentally stable nonenveloped viruses that are resistant to disinfection; (2) the frequent and growing exchange among investigators and institutions of genetically engineered mutant mice harboring pathogens; and (3) the use of research animal-derived reagents and cell substrates in the development and production of parenterally administered biopharmaceuticals. The key factors determining the likelihood that HM findings correspond to the pathogen status of the principal animal population are the accuracy of the assay results and the degree to which samples are representative and suitable for the diagnostic methodologies by which they are tested. This section will review these factors, with emphasis on the challenges to obtaining accurate HM results for rodents housed in microisolation caging systems, including the typically low prevalence of infection and reliance on soiled bedding sentinel monitoring, and the role that PCR is playing in addressing these challenges by enabling testing of pooled environmental and *ante mortem* animal specimens not suitable for traditional methodologies.

1. Assay Accuracy

The key indicators of accuracy for primary surveillance tests are diagnostic sensitivity (DSe) and specificity (DSp), i.e., the proportions of correctly classified known positive and negative samples, respectively (reviewed in Jacobson, 1998; OIE, 2013; Pepe, 2003; Tyler and Cullor, 1989; Zweig and Robertson, 1987). Estimates of DSe and DSp are obtained by testing known positive and negative samples from pathogen-infected and pathogen-free animals, respectively, and by comparing an assay's results to those of a 'gold-standard' reference test. For serologic and PCR assay methods that yield numeric titers or instrument readings such as optical density or fluorescence intensity, sample results are classified as positive (+), negative (−), or equivocal (+/−) compared to positive and negative cutoff values, as shown in Fig. 11.9. Raw instrument readings may be used as the result values, but a common practice is to calculate values from one or more instrument readings in order to simplify the examination and classification of test results by (1) reducing the number of digits and decimals, (2) subtracting background so that negative result values are near zero, and/or (3) normalizing values, e.g., scoring sample readings in comparison with the positive control for the assay run. In addition, a sample result may be classified as indeterminate when a sample suitability control fails because the sample reacted nonspecifically or was inhibitory, or its quantity or target concentration was insufficient. Examples of the system and sample suitability controls utilized for serology by MFIA and for PCR assays are shown in Table 11.5. Sample results should only be classified (and reported) if the standard positive and negative system suitability control results were satisfactory, thereby demonstrating that assay sensitivity and specificity were acceptable. Thus, including the appropriate system and sample suitability controls in each assay run is essential to diagnostic accuracy.

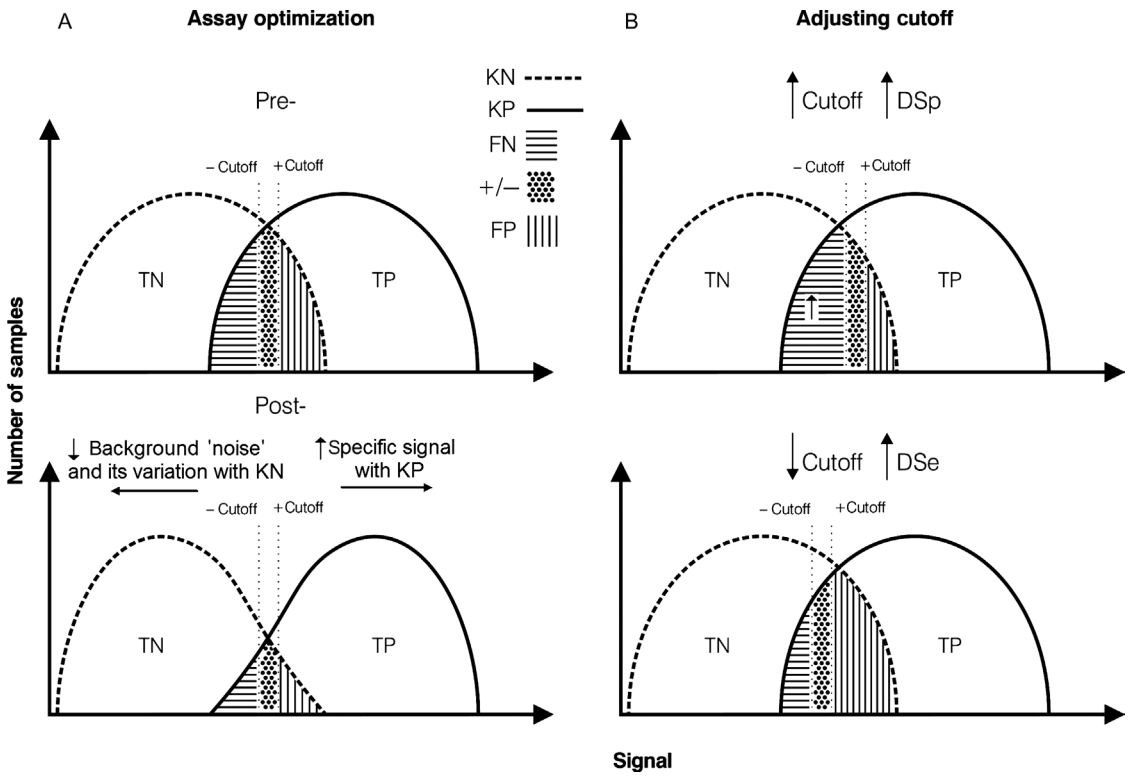

FIGURE 11.9 Classification of assay results. Serologic and PCR assays yield numeric titers or instrument readings such as optical density or fluorescence intensity. These numeric data, or values calculated from them, are classified as positive (+), negative (−), or equivocal (+/−) by comparison to positive and negative cutoff values, which are determined by testing known positive (KP) and known negative (KN) samples from pathogen-free and infected animals. Diagnostic sensitivity (DSe) and specificity (DSp) are the percentages of KP and KN samples correctly classified as true positive (TP) and true negative (TN), respectively. The schematic graphs in panel A depict the distribution of results for KP and KN samples tested by a hypothetical assay 'pre-' and 'post'-optimization. The goal of assay optimization is to reduce the percentages of samples that yield false-negative (FN), false-positive (FP), or +/− results by increasing the specific signal given by KP samples and decreasing the background 'noise' level and variation for KN samples. Following optimization, as shown in panel B, cutoffs can be adjusted to favor DSe or DSp depending on whether FN or FP determinations are more problematic. For example, cutoffs may be increased to favor DSp and avoid FP findings in tests for rare pathogens or decreased to favor DSe and avoid FN when testing for common pathogens.

Cutoff values are chosen initially based on analysis of data from assay development and validation; subsequently, they may be adjusted in accordance with the results of routine quality control and surveillance. Receiver operating characteristic curve analysis, comprising various graphs and statistics to summarize the DSe and DSp for a range of cutoffs values, is a common methodology for empirically evaluating the optimality of cutoffs (Greiner *et al.*, 2000; Pepe, 2003; Zweig and Campbell, 1993). Cutoffs can be adjusted to favor DSe or DSp depending on whether FN or FP determinations are more 'costly'. Assay cutoff values might be increased to favor DSp and avoid FP results in tests for rare pathogens; conversely, they may be decreased to enhance DSe when testing for common agents to reduce the likelihood that adventitious infections will be missed due to FN findings (Fig. 11.9B).

The basic strategy for optimizing the diagnostic accuracy of a primary surveillance test is to decrease the 'noise', i.e., the strength and variation of the background signals given by known negative samples from pathogen-free animals, and to boost the specific signal or titer given by known positive samples from infected animals, thereby reducing the number of samples with signals or titers near the cutoffs that are most likely to be FN or FP (Fig. 11.9A). The amount of separation between positive and negative signals is dependent on an assay's analytical sensitivity and specificity. The former is measured as the limit of detection (LOD), which is the lowest concentration of a target analyte (e.g., antibody, organism, or genomic sequence) in a specific matrix (e.g., serum, tissue homogenate, or feces) that can consistently yield a positive result. For PCR, samples can be 'spiked' with a known number of template copies to determine LOD; however, as quantifying the actual concentration of polyclonal serum antibodies to multivalent microbial antigens is not feasible, the LOD of serologic immunoassays is usually presented as a comparison of titration endpoints for an antiserum by two or more tests. The analytical sensitivity needed for a surveillance assay

TABLE 11.5 System and Sample Suitability Controls for Serology by the MFIA and for the PCR Assay

Method	Type	Qualification of	Assay for	Sample	Satisfactory
MFIA	System[a]	Analytical sensitivity	Microbial antibodies	Control-positive (C+) standard (std) antiserum	+
		Analytical specificity	Microbial antibodies	Control-negative (C−) std SPF serum	−
			Microbial antibodies	Diluent (Blank)	−
		Assay procedure (process)	Microbial antibodies	Std serum processed with test (i.e., noncontrol) samples	+ or −
			Labeled anti-species IgG probe	BiotinαIgG, phycoerythrin-streptavidin	+
	Sample[b]	Low nonspecific background	Nonspecific antibody binding	Test serum	−
		Adequate antibody quantity	Serum total IgG	Test serum	+
PCR	System[a]	Analytical sensitivity	Microbial template	Positive template control (PTC)	+
		Analytical specificity	Microbial template	Negative template control (NTC)	−
		Procedure: extraction and RT[c]	RNA template spike recovery	Exogenous RNA template	+
		Procedure: sample processing	Mock extraction	None or NTC	−
	Sample[b]	DNA recovery and purity (i.e., lack of PCR inhibitors)	PTC template Internal host cell template	Test specimen + PTC spike added to PCR Test specimen	+
		Recovery: RNA from extraction and cDNA from RT step	RNA template spike recovery	Test specimen + RNA template spike added to sample before extraction	+

[a]*System suitability controls qualify the overall performance of the assay run; the most commonly employed of these controls are the C+ and C−, which are tested to verify that analytical sensitivity and specificity, respectively, are satisfactory; others confirm that the procedure steps are being carried out and are performing properly, i.e., labeled anti-species IgG has been added to test wells or that cross-contamination (of the PCR mock control) did not occur during nucleic acid extraction. All samples in a test run with one or more failed system suitability controls should be retested.*

[b]*Sample suitability controls assess the fitness of the sample for the assay methods. For example, if a test serum gives a high background signal in the tissue control test for detection of nonspecific binding of serum antibodies to the solid phases, a positive signal in a microbial antibody MFIA is classified as indeterminate because it could also be nonspecific; a specimen negative by PCR is reported indeterminate if the PTC spike control reaction is negative, indicating that the sample is inhibiting the PCR.*

[c]*RT = reverse transcription of viral genomic RNA into complementary DNA by a reverse transcriptase enzyme (often originating from a retrovirus). This step is necessary for RNA viruses because the PCR DNA polymerase cannot directly copy RNA templates.*

depends on the expected range of concentrations of target analyte in specimens and the volume of sample tested. For example, a 'moderate' level of sensitivity suffices for the immunoassays used to screen for common viruses because these viruses typically elicit a strong antibody response in immunocompetent sentinels. By contrast, the extreme analytical sensitivity of PCR assays is important to their DSe because it allows detection of the low pathogen concentrations (or more correctly nucleic acid template copy number) expected in heavily pooled (e.g., 10:1) animal and environmental specimens; conversely, extreme analytical sensitivity can negatively impact the DSp of PCR when amplification of small quantities of template not associated with an active infection yields FP findings. As already mentioned and reviewed in the next section, sample selection errors, such as performing serology on acutely infected or PCR on convalescent hosts, can reduce the concentration of the targeted analyte below the test LOD.

Analytical specificity is defined as the ability of an assay to distinguish target from nontarget analytes, including matrix (i.e., specimen) components; it comprises selectivity, inclusivity, and exclusivity, (OIE, 2013). A selective assay is one with the capacity to resist matrix-mediated effects such as those detected by sample suitability controls including sample-mediated inhibition of the PCR, nonspecific binding of the serum antibodies (or labeled anti-Ig) to the solid phase, and target analyte degradation. Inclusivity refers to the ability of a single test to detect related organisms of interest, whereas exclusivity describes a test able to differentiate an infectious agent from others that are closely related. As a general strategy, inclusive assays are preferred for primary testing because they can reduce both the chance of missing an adventitious infection and the number of assays needed for surveillance. For example, lab animals are commonly screened for *Helicobacter* infection by a single inclusive PCR test that detects all species of the *Helicobacter* genus.

Exclusive assays, however, are important for confirmatory testing to corroborate preliminary findings and to determine the species, strain, serotype, or genotype of an adventitious agent when it is relevant to the SPF status of the principals or useful for investigating an outbreak. In the case of *Helicobacter*, additional testing by a number of species-specific PCR tests is carried out to corroborate initial findings and because the *Helicobacter* species can affect the course of action. For instance, certain species, such as *H. hepaticus* and *H. bilis*, are considered to be more pathogenic and likely to interfere with research than others (Drazenovich *et al.*, 2002; Henderson *et al.*, 2013; Whary and Fox, 2004). An additional example of the importance of sequencing is derived from an investigation of murine rotaviruses recovered from concomitant outbreaks at multiple institutions. The agents had identical genotypes, and repeated testing that included genomic sequencing provided convincing evidence that linked the outbreaks to a common source of contaminated bedding (Dole *et al.*, 2013a).

The analytical specificity of serologic and PCR assays is chiefly determined by the antigen bound to the immunoassay solid phase and the genomic sequences to which the primers (and probe for qPCR) anneal, respectively. Antigen for ELISA or MFIA may consist of organisms purified to varying degrees, organism extracts, or recombinant proteins, which have facilitated the development of assays with predictable analytical specificity. For example, ELISA and MFIA for viral antibodies that utilize a recombinant protein antigen conserved among related viruses, such as the nonstructural NS1 protein of parvoviruses (Riley *et al.*, 1996b), the VP6 capsid of group A rotaviruses (Zhu *et al.*, 2013), and the nucleocapsid NP protein of LCMV (Homberger *et al.*, 1995), are inclusive, whereas those employing recombinant envelope or capsid protein antigen that possesses neutralizing epitopes, such as the parvovirus capsid protein VP2 capsid protein (Henderson *et al.*, 2015; Livingston *et al.*, 2002), are exclusive (i.e., primarily detect serotype specific antibodies). IFAs for viral antibodies are intrinsically inclusive because they typically use as antigen-infected cells containing all virally encoded proteins. By detecting antibodies to conserved nonstructural proteins, rodent parvovirus IFA provided the initial evidence in the 1980s for the existence of parvovirus serotypes in addition to those represented by the prototypical strains used at the time in serotype-specific HAI tests (Jacoby *et al.*, 1996). Inclusivity achieved with complex antigen consisting of whole organisms, infected cells, or crude extracts of those, however, can increase the rate of false-positive findings when antibodies bind to nonmicrobial constituents of the antigen preparation or to antigenic determinants shared with commensal microbes; moreover, raising the positive cutoff to compensate for higher background is likely to reduce DSe. Relying on a single conserved recombinant protein antigen can also compromise DSe when the antigen is not consistently immunogenic or elicits an antibody response that is weak or delayed, as has been demonstrated for the rodent parvovirus antibody response to NS1 *vis-à-vis* capsid proteins.

PCR assays are made inclusive or exclusive by designing primers (and probes for qPCR) that anneal to genomic sequences, which are shared by or unique to variants of the agent of interest, respectively. Designing primers that fulfill these criteria and amplify a small product (e.g., under <200 bp) for optimal sensitivity can be demanding when the targeted pathogen species or group is very heterogeneous or there is a paucity of DNA sequence information available for commensal and other extraneous organisms likely to be present in clinical specimens. These challenges have been eased by access to increasingly sophisticated and cost-effective tools, techniques, and services for DNA sequencing and analysis that have expedited the identification of conserved and differential genomic sequences for PCR amplification in a wide variety of infectious agents. PCR (and probe hybridization) targets are generally more plentiful than those that can be distinguished by phenotypic tests because (1) they can be located in regions of the microbial genome that do not encode proteins and (2) the sequence of a gene often varies more than the amino acid sequence of the protein it encodes due to the degeneracy of the genetic code (i.e., multiple codons are translated into the same amino acid). In addition, the analytical specificity of genetic targets are unaffected by environmental and host-related factors that can alter the phenotype of an organism or humoral immune response to infection. Thus, the analytical specificity of PCR assays by and large exceeds that achievable by traditional tests.

The probability that a positive or negative result is correct is referred to as its predictive value. Besides being a function of assay DSe and DSp, predictive values are substantially affected by the prevalence of infection. The positive predictive value (PPV), which is the percentage of all positive results that are true positive, decreases along with the prevalence (Laregina and Lonigro, 1988; OIE, 2013; Zweig and Robertson, 1987). When the prevalence of infection is very low, as has been found for adventitious infections of animals housed in microisolation cages, a substantial percentage of positive results are expected to be FP, even for assays with DSp approaching 100%. The example shown in Fig. 11.10 illustrates that for an assay with DSp and DSe that are 99%, dropping the prevalence of infection from 50% to 2% decreased the PPV from 99% to 67%; to put it another way, one-third of the positive results are expected by FP when the prevalence of infection is 2%. This underscores the need, emphasized throughout this chapter, for repeat testing to corroborate new positive findings.

Number of Samples Tested: 10,000 **DSe:** 99% **DSp:** 99%

Value		Formula	Prevalence 50%	Prevalence 2%
Samples	KP:	# Tested × Prevalence	5000	200
	KN:	# Tested × (1−Prevalence)	5000	9800
Positive results	TP:	KP × DSe	4950	198
	FP:	KN × (1−DSp)	50	98
	Total:	TP + FP	5000	296
			99%	**67%**
	PPV:	TP/(TP+FP)		
			1% FP	**33% FP**

FIGURE 11.10 Effect of prevalence on PPV. The PPV is the percentage of all positive results – including true-positive (TP) and false-positive (FP) results given by known positive (KP) and known negative (KN) samples, respectively – that are TP. For an assay with diagnostic sensitivity (DSe) and specificity (DSp) both equal to 99%, dropping the prevalence of infection from 50% to 2% decreased the PPV from 99% to 67%. That one-third of the positive results for this highly accurate assay are expected to be FP when the prevalence is 2% underscores the critical importance of repeat testing to corroborate new positive findings.

2. *Sample Selection*

The overall goal of an HM program is to obtain results that are reflective of rodent colony health status. In addition to the influence of test accuracy, the correspondence of HM findings to the actual health status of the population being monitored is affected by the degree to which the samples are representative of the population and specimens are suitable for the tests employed. For commercial barrier rooms, HM is performed directly on colony animals of both sexes and multiple age groups. Commercial breeders may house their animals in open-topped cages to promote rapid and unimpeded spread of infection by all modes. Aerosols are generated that fall into other cages, as do bedding and feces from nearby cages. Animals are transferred among cages for breeding and stocking, and technicians manipulate multiple cages in succession. All of these factors promote transmission and, therefore, early detection of any microbial contamination. In addition, commercial breeders monitor their colonies on an almost continuous basis, in contrast to academic and other institutions that usually monitor on a quarterly schedule.

a. Principal Animals

Using resident colony animals for HM avoids the chance of introducing unwanted infectious agents that might be present in sentinel animals. Additionally, there is no time lag as occurs with exposure of externally sourced sentinels; whenever a sample is needed, one merely selects the desired number of animals directly from the population in question. However, use of resident animals for HM involves certain assumptions. One assumption is that the animals selected for monitoring have been adequately exposed to any infectious agents present. Exposure may be accomplished through husbandry practices, through the use of open-topped cages, or through transfer of potentially contaminated fomites such as soiled bedding. Sampling colony animals that are housed in individually ventilated cages without additional exposure such as through transfer of soiled bedding may be unlikely to test positive for any infectious agents present, as only a very small percentage of cages may be contaminated (Shek and Gaertner, 2002; Shek *et al.*, 2005). Another assumption is that all animals in the population are genetically susceptible to infection with the agents being monitored and the panoply of inbred strains and genetic modifications in contemporary mouse populations militates against that.

Animals selected from the colony for serologic monitoring must be immunocompetent and therefore able to produce antibodies to viral or microbial agents. Immunocompetence may be difficult to determine or unknown in some genetically engineered animals, as there are numerous anecdotal reports of incomplete immune responses in genetically engineered mice previously thought to be immunocompetent. By contrast, intentional use of immunodeficient animals may enhance the sensitivity of surveillance relying on direct detection of infectious agents, such as PCR, bacteriology, or parasitology, because immunodeficient animals

may sustain infectious agents for long periods, if not indefinitely (Besselsen *et al.*, 2007; Compton *et al.*, 2004b; Henderson *et al.*, 2012; Macy *et al.*, 2013).

Selecting resident animals of the appropriate age is more problematic than selecting the appropriate age of sentinel animals. In general, the oldest animals in a population are the most likely to have encountered (and seroconverted to) an infectious agent during their tenure. However, immunocompetent animals mount a host response and partially or entirely clear many parasitic, microbial, and viral infections, so that aged animals are less likely to test positive by direct examination and cultural isolation. In enzootically infected colonies in which newborns are protected by maternal antibodies, recently weaned to young adult animals are those most likely to be heavily infested with protozoan and metazoan parasites and actively infected with viruses and microbial pathogens, such as *C. piliforme* and *P. carinii* (An *et al.*, 2003; Henderson *et al.*, 2012; Waggie *et al.*, 1987), that are cleared by the host adoptive immune response.

In theory, the number of animals tested is based on the predicted prevalence of the infectious agent in the colony (Anonymous, 1976; Clifford, 2001; Dubin and Zietz, 1991; Selwyn and Shek, 1994). For barrier room colonies housed in open-topped cages, which are monitored by sampling of colony animals, this means that eight animals are sampled at each testing interval. This gives a 95% probability of detecting a pathogen when it reaches a prevalence of approximately 30% in the population; experience has shown that recently introduced viruses actually reach a prevalence above 50% within a single 4-week monitoring interval (Selwyn and Shek, 1994; Shek *et al.*, 2005). For greater security, this type of facility may (and frequently does) choose to monitor more animals and/or sample more frequently. Today, a majority of research rodents are housed in static or ventilated microisolation cages where the prevalence of an agent is kept low by the very nature of this type of housing. If an agent were present at a 10% prevalence, 30 animals would need to be screened for a 95% chance of detection, and if an agent were present in only 1% of animals, then 300 animals would need to be screened (Clifford, 2001). Prevalence as low as 2% has been considered realistic for MPV infection of mice in IVC (Macy *et al.*, 2009; Smith, 2010). Because sampling of sufficient numbers of mice to detect such low-prevalence infections is not feasible, most facilities use testing of sentinel animals.

b. Sentinel Animals

In reality, principal animals being used in research are rarely made available by scientists for blood draws or euthanasia for conventional HM, and consequently, colonies are monitored indirectly by testing sentinels. Sentinel animals are generally externally sourced animals that are introduced into a population, exposed to animals or soiled bedding from the population, and sampled in lieu of the principal animals. Sentinels should be immunocompetent so that they are suitable subjects for serology, and should have a mature immune system when sampled. Outbred stocks are recommended because they are generally good serologic responders and are usually less expensive. Conversely, inbred sentinels should be avoided because some inbred strains have been shown to be comparatively resistant to infection with or to mount a delayed antibody response to certain pathogens (Besselsen *et al.*, 2000; Brownstein *et al.*, 1981; Drake *et al.*, 2008; Henderson *et al.*, 2015; Hirai *et al.*, 2010; Shek *et al.*, 2005). Sentinels should be female, which will decrease fighting within the sentinel cage and lessen the chance of genetic contamination of the principal animals from contact sentinels. They should of course be free of all infectious agents, which would be of concern for the principals being monitored. If those agents include opportunistic pathogens, as might be the case for immunodeficient models, then sentinels should be obtained from gnotobiotic or SOPF colonies raised in isolators rather than barrier rooms. As mentioned, sentinels are externally sourced from commercial vendors because this is considered to be simpler and less expensive than attempting to produce clean sentinels internally. If, however, sentinel animals are bred at the facility, they should be monitored at an increased frequency, perhaps monthly, as they are a potential source of infection for the entire facility. However, there is a biosecurity risk associated with the routine receipt of sentinels from commercial vendors that is low but not zero (Pullium *et al.*, 2004; Shek *et al.*, 2005).

The current standard practice for routine surveillance of resident animals is to keep sentinels in separate cages supplied with regular changes of soiled bedding pooled from colony cages. Typically, one or two sentinel cages are set up for a rack of cages (frequently one cage per side of a rack). Contact sentinels are impractical for routine surveillance for several reasons, the most important being their potential to contribute to the spread of infection. Reliance on soiled bedding alone to transmit infections to sentinels, however, is problematic because infections with certain respiratory viruses, host-adapted bacteria, and parasites are transmitted inefficiently or not at all in soiled bedding (Artwohl *et al.*, 1994; Compton *et al.*, 2004c; Cundiff *et al.*, 1995; Dillehay *et al.*, 1990; Henderson *et al.*, 2013; Ike *et al.*, 2007; Lindstrom *et al.*, 2011; Thigpen *et al.*, 1989). In addition, the ability of microisolation cages to control the spread of infection frequently keeps the percentage of cages with actively infected rodents low, thus presenting challenges for detection by the HM program. Irrespective of the test methodology used, detection of transmission to sentinels is dependent on exposure of those animals to an

infectious dose of the microbial contaminant. Thus, the lower the prevalence of infection, the greater the risk that the pathogen dose in pooled bedding will not be sufficient to infect sentinels. As cited above, that risk may increase as sentinels become older and less susceptible to infection with certain agents. However, a recent study demonstrated that the transmission of common adventitious viruses (including MPV) and pinworms to sentinels via soiled bedding did not differ according to whether the sentinels were weanlings, young adults, or aged (Grove *et al*., 2012).

c. General Principles of Sentinel Exposure

Sentinels should be exposed to new soiled bedding at each cage change, keeping in mind that the shedding of many pathogens eventually stops. As much soiled bedding as possible should be placed in the sentinel cage to minimize the diluting of infectious material; most facilities transfer 5–15 ml from each principal cage. Clean bedding should not be placed in a sentinel cage because it further dilutes infectious agents in the soiled bedding, possibly to concentrations below the infectious dose for the sentinels (Besselsen *et al*., 2008a; Smith *et al*., 2007). The placement of new nesting material into the sentinel cage is a reasonable compromise and is recommended wherever this enrichment method is in use. Sentinels should be given a period of at least several weeks to become infected and seropositive, but since HM at research facilities is done quarterly or less often, sentinels are exposed for a minimum of 3 months before being tested.

Depending on the required health status of the principal animals and the types of organisms to be excluded as well as the type of husbandry and housing systems used, the composition of the sentinel cage group(s) and the number of sentinel animals per cages may vary. Typical recommendations are that one sentinel cage of two to three sentinels be used for monitoring a 50–80-cage rack or side of a rack, but often additional sentinel cages are used to increase the amount of soiled bedding which can be sampled from each principal cage. Housing more than one sentinel per cage is beneficial when unexpected deaths occur or when a cage mate is required to confirm an unexpected positive result. Risks and costs should be balanced by the institution. For example, finding an unexpected positive might require individual testing of 50–80 colony cages versus the cost of monitoring two sentinel cages and then individually testing 25–40 colony cages, should there be a positive.

An economical alternative to testing all sentinels in a cage for all agents is to test only one per cage (or to test a pool of *ante mortem* specimens). At the time those samples are submitted, new sentinels can be added to the cage as a 'bridge' for any recent infections or exposures to which the previous sentinels had not yet seroconverted. When the results from the submitted samples are received, the remaining 'old' sentinels can be euthanized, can be used for confirmation of any positive findings, or can be used to sample for agents of particular concern, e.g., any present in the facility, or even for a full panel. It should be noted that occasionally not all animals in a cage will seroconvert to MPV, so testing only a subset of the sentinels slightly decreases the sensitivity of the sentinel program. However, facilities may accept this slight decrease in sensitivity in exchange for the cost savings, or they may choose to test the second sentinel only for parvoviruses. Alternatively, mesenteric lymph nodes can be collected for PCR testing as a supplement to MPV serology.

C. Health Management Program Management

HM programs should be designed by a knowledgeable person, based on the needs of the facility/institution, and subject to frequent review and updates as necessary. It is important to remember that the goal of an HM program is not necessarily to exclude all possible infectious organisms, but rather to monitor for the presence or absence of a select set of organisms that have the potential to affect research conducted in that facility. A positive for an infectious agent on a health report does not necessarily mean that the facility should be depopulated or that the animals are not usable. While recommendations for exclusion lists exist (Guillen, 2012; Nicklas *et al*., 2002), those recommendations should always be interpreted by someone with knowledge of the needs of the facility or institution. Financial constraints may also play a role in decisions regarding exclusion; for instance, a large institution with rodents housed in multiple facilities, all of which are positive for *Helicobacter* spp., may decide to eliminate it from a subset of those facilities, depending on the needs of scientists using the animals. Thus, detection of an agent could result in immediate eradication, planned eradication, or acceptance, depending on research goals.

HM programs should be as simple in design as possible and any components that can be computerized, especially for large programs, will help in simplification. Factors to consider at the earliest stages include the host species that will be monitored (including immune function and pathogen status); the source of animals that will be monitored (all from approved commercial breeders or nonapproved sources, such as academic institutions, or a combination of the two); how the animals are used (e.g., breeding and distribution or research); and biosecurity level available at institution (barrier or nonbarrier and/or static or ventilated microisolation cage, isolators, or a combination). If animals will be obtained from nonapproved sources, it is critical to consider the availability of a space in which incoming animals can be quarantined prior to introduction into existing colonies,

keeping in mind that these animals are not transported in dedicated vehicles.

1. Design and Implementation

Large and complex academic programs tend to be geographically dispersed and have vivaria that cater to many research needs. These include barrier facilities from which rodents make 'one-way' trips (e.g., breeding colonies), conventional facilities that may permit animals to be transported to laboratory space with subsequent return to the vivarium, and specialized housing for rodents used in behavioral testing, sequential imaging procedures, or metabolic assays.

Designing an HM program for such diverse housing and research modalities can be daunting. As a first step, the program should have universal features that are applied to all vivarial situations. These can be customized to meet the needs of the research performed in the various types of housing modalities; however, customization should probably be minimized so that the simplicity suggested above is retained, resulting in lower likelihood of human error. An animal resource leader knowledgeable about institutional needs and financial constraints must decide on methodologies to be used (e.g., serology, culture, physical and microscopic examinations versus PCR); testing intervals (quarterly, semiannually, annually); test panels (basic versus comprehensive); animals/materials to be tested (soiled bedding or contact sentinels, principals, environment); and samples to be collected (serum or DBS, feces and swabs (from animals or environment), or live animals). In considering methods, one must keep in mind that serology and PCR yield different information, the former simply indicating infection at some prior, undetermined time and the latter indicating the current infection or nucleic acid presence. Based on scientific needs, an exclusion list should be established and a plan devised for actions taken when an excluded agent is detected (immediate versus 'planned' eradication).

Testing is usually performed quarterly, after full exposure of the sentinels to all cages of the sampling area. Sentinels should not be kept more than 6 months as their sensitivity to certain excluded pathogens may decrease as they age (Riepenhoff-Talty *et al.*, 1985). However, sentinels may take months to become infected and seroconvert (Henderson, 2008). Thus, quarterly rotation and testing of sentinels seems to best fit the optimal time window for serologic detection of the most prevalent infectious agents.

For routine HM, most facilities choose to monitor quarterly as the default, with increased frequency of monitoring used to assist disease eradication or containment efforts for particular agents. A frequently used paradigm includes testing three times each year for the most prevalent agents (those that are most likely to be introduced into a vivarium), and annually for a more comprehensive panel of infectious agents. The comprehensive panel is employed to satisfy import requirements of collaborating institutions, to address any inquiries by regulatory bodies, and to detect infections which are rare in contemporary lab animals but still common in wild and pet rodents, and which may also be present in materials archived in freezers from the days when more viral and bacterial infections, including some zoonoses, were prevalent.

Once the details of the program designs have been established, programs are implemented by assigning them to locations and colonies, and by generating a master HM schedule for collecting and submitting samples (Table 11.6). For a campus managing multiple vivaria with each housing variable numbers of rodents, the schedule can be adjusted to even-out the workflow for the HM technicians over the course of a year. Simple modifications, such as PCR testing of feces for MPV in barrier-maintained sentinels, can be easily inserted into the master schedule without too much concern that this

TABLE 11.6 HM Program Design and Implementation

STEPS
DESIGN
Develop specifications, i.e., lists of excluded and other reportable agents, by species, immune status, barrier system, source, use, etc.
Determine frequency of testing for each agent according to risk and cost of contamination
Select/create/customize test panels by species, diagnostic methodology, and frequency of testing for specific agents
For each panel, choose sample and specimens types that are suitable for the HM methodology and determine the sample number.
By program, arrange protocols (i.e., panels + samples) in the appropriate order, separated by intervals corresponding to the frequency of testing.
For results summarization, define diagnoses and the laboratory tests they comprise.
IMPLEMENTATION
List animal locations (typically by facility, building, and room) and colonies (which can be actual colonies or monitoring units such as a cage racks).
Assign HM programs to colonies.
Generate master schedule indicating the location, colony, collection date, protocol, and testing laboratory.
Collect and submit samples according to schedule.
Review and file reports.
Maintain 12–18-month longitudinal summary of results (as positive/tested) by species, location, and colony.

will be forgotten by the HM technicians packing and submitting the animals.

Although we have emphasized serologic and direct sampling monitoring of sentinel animals in this section and in the accompanying table, there is an emerging trend away from sentinel use and toward environmental monitoring as a way to better sample principal animals that are housed in ventilated caging systems. Such non-sentinel monitoring also has the advantage of detecting agents that are difficult or impossible to transmit via soiled bedding (Artwohl *et al.*, 1994; Compton *et al.*, 2004c; Cundiff *et al.*, 1995; Dillehay *et al.*, 1990; Henderson *et al.*, 2013; Ike *et al.*, 2007; Thigpen *et al.*, 1989) and it supports the 3Rs principles.

2. *Results Analysis and Summarization*

Data generated by an HM program should be reviewed by an individual at the institution who understands the biology of the agents and the scientific goals of the rodent users and is responsible for the overall biosecurity program. This person should examine the data for anomalous or unexpected findings and then initiate appropriate action. Actions might include report filing or data compilation if no actionable results are received, communication with the testing laboratory for all unexpected or actionable results, or notification of other responsible individuals at the institution. Because of the significant consequences of many infections and the equally significant consequences of erroneously taking action in situations where no infection exists, the first step should always be to contact the testing laboratory prior to executing any eradication plan or other research-inhibiting actions. The testing laboratory cannot only give some idea of the level of confidence in the results (i.e., was it a strong positive versus near-threshold result? Does that particular assay often give FP results? Is the positive predictive value of the result low because that particular agent is very rare?), but can also help plan the quickest and most definitive confirmation testing.

Health reports that require the reader, often the import coordinator at a potential recipient institution, to sift through many pages to identify the room or vivarium of interest are not useful and generally end up as reams of wasted paper, even if recycled. The results of HM testing should be summarized into a one- or two-page HM report for areas within institutions (usually for individual vivaria at academic institutions). This allows for easier interpretation for import/export of animals and less confusion by shipping and receiving institutions as to the agents tested and results obtained. A useful health report does not only give results from the most recent and historical (up to 18 months) testing but must also provide additional information about housing and maintenance procedures, any treatment provided, and the HM program and exclusion list (Nicklas *et al.*, 2002) (Table 11.7). It is also important to provide contact information for the person responsible for the HM program in the animal facility in case additional information is requested. Not all of these data are easily provided on a health report, and ideally institutions should also provide a one- to two-page HM program description with the HM results. It should be emphasized that a high-quality HM program is of utmost importance to institutions exporting large numbers of rodents. Having a reputation for outsourcing infectious disease outbreaks is not a winning strategy.

TABLE 11.7 HM Summary Report Information

Results	Diagnosis[a]
	Primary assay
	Most recent # Positive/# Tested
	Longitudinal # Positive/# Tested
	Longitudinal period in months
	Frequency of testing by diagnosis
	Frequency of testing by protocols[b]
Colony	Institution location
	Species, strain
	Specification (e.g., SPF, SOPF)
	Barrier system
	Treatments
	Other colonies in location

[a] *An infectious agent, based on assays performed in one or more laboratories.*
[b] *Protocols include the laboratory, assay panels, and samples (e.g., principals, sentinels, exhaust air dust, etc.).*

V. OUTBREAK MANAGEMENT AND INVESTIGATION

A. Repeat Testing to Corroborate New Positive Findings

As the prevalence of infection decreases, so does the PPV. When the prevalence of infection is very low, as is frequently the case following contamination of rodents in microisolation cages, a substantial percentage of positive results are likely to be FP, even for assays having diagnostic accuracy (i.e., DSp and DSe) near 100% (Fig. 11.10). In addition, irrespective of diagnostic accuracy, no assay is immune to sample selection errors (Table 11.8) and mistakes made in the laboratory including sample mix-ups, noncompliance with SOPs,

TABLE 11.8 HM Animal Sample Selection Errors

Principals/ HM	Methodology	Animals sampled
Infected/ HM-negative	Serology	Acutely infected, prior to seroconversion Immunodeficient, unable to mount antibody response
	PCR/ bacteriology/ parasitology	Older and recovered from infection From sites where organism is not resident or target concentration is below the test LOD
	All	Too few in number for low prevalence of infection Uninfected sentinels because Prevalence of infection in principals was low Pathogen not transmitted via soiled bedding Resistant to infection due to age or genetic background Unintentionally from the wrong colony or samples mislabeled postcollection
Uninfected/ HM-positive	Serology	Tissue reactive antibodies because of Age or autoimmune disease Inoculated with biologic Elevated levels of serum Ig due to age Maternal antibodies
	PCR	Were negative, but samples from them were contaminated during collection or processing Low levels of template not associated with active infection (e.g., ingested noninfective template from the environment)
	All	Were sentinels infected from extraneous, i.e., noncolony source, e.g., while in transit Unintentionally from the wrong colony or samples mislabeled postcollection

data calculation and transcription errors, incorrect interpretation of findings, and so forth. Therefore, we cannot overemphasize the importance of confirming new positive findings – even those made by direct examinations that would seem irrefutable – before undertaking disruptive and costly actions to eradicate an infection. When one considers that most programs rely on quarterly testing and an adventitious infection is likely to require at least several weeks to spread to and reach a detectable level or elicit seroconversion in sentinels, it is reasonable to expect that a period of a month or longer has elapsed between the occurrence and detection of an outbreak. Thus, taking few a more days to a week to corroborate initial findings poses little additional risk to other animals in the facility, especially if the animals are maintained in microisolation cages that provide biocontainment.

Confirmatory testing, employing when feasible alternative assay methods and target analytes (i.e., antigens for serology or genomic sequences for PCR) as well as complementary methodologies, is frequently performed concomitantly on samples from the sentinel cage mate(s) and resident animals on the suspect side(s) of the rack monitored by the positive sentinel(s). Confirming infection of resident animals is worthwhile because sentinels may have acquired the infection from an extraneous source prior to placement, e.g., while in transit or quarantine. In addition, environmental specimens such as swabs of cages or the exhaust air dust from ventilated rack plenums or filters can be submitted for molecular testing (Jensen *et al.*, 2013). The rule of thumb at one author's institution is that positive samples detected using commercially available antigen-coated ELISA plates are retested by IFA using cells infected with virus from different sources. Even when both tests are positive, the suspect sample is shipped to a commercial lab that runs MFIA with additional antigens and IFA using further, independently prepared reagents.

Determining the appropriate assay methods and specimens for repeating testing should be based on the knowledge of the adventitious agent's pathobiology including the time course and sites of infection and shedding, and the time to seroconversion. For instance, a murine rotavirus seropositive sentinel might lead to testing its cage mate by fecal PCR. A negative PCR result would not clarify the accuracy of the earlier result. This is because murine rotavirus causes an acute, self-limiting infection with shedding and transmission ceasing at some point after antibody development (Riepenhoff-Talty *et al.*, 1985). More useful follow-up data could result from serologic testing of the cage mate and/or PCR on fecal pools of the relevant colony population or on IVC exhaust. In the case of an MPV seropositive sentinel whose cage mate was subsequently found to be negative by fecal PCR, additional testing would be required. MPV shedding and transmission wane after seroconversion, but viral DNA persists in selected tissues, notably mesenteric lymph node (Besselsen *et al.*, 2007; Henderson *et al.*, 2015; Jacoby *et al.*, 1995; Shek *et al.*, 1998; Smith *et al.*, 1993a). If PCR on mesenteric lymph node yielded a negative result, one could infer that the serologic finding represented a false positive. Because MNV continues be shed at high levels in the feces of seropositive mice, negative PCR results for feces from a seropositive sentinel and/or its cage mate(s) provide significant support for the serology being FP. The patterns of infection and shedding in relation to seroconversion

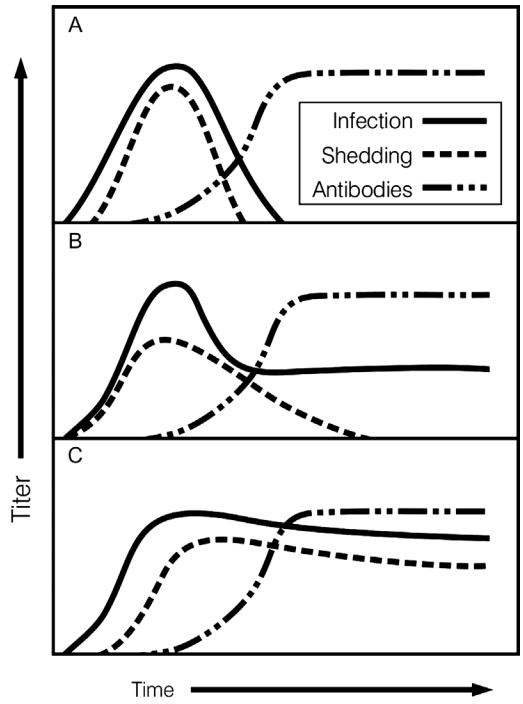

FIGURE 11.11 Basic patterns of infection and shedding in relation to seroconversion. Following seroconversion, an infectious agent may be cleared from (A) or persist in (B, C) host tissues; for a persistent infection, shedding of the organism may wane rapidly (B) or continue for a prolonged or indefinite period (C). As discussed at the beginning of Section V, knowing the pattern that an infectious agent follows is crucial when carrying out repeat testing to substantiate new positive findings.

by immunocompetent hosts just described for murine rotavirus, MPV, and MNV are schematically depicted in Fig. 11.11A, B, and C, respectively.

B. Containment and Eradication

Once the presence of an excluded agent has been confirmed, the outbreak must be contained and managed. At academic institutions that generally provide scientists with 24/7 vivarial access, containment can be a challenge. Although investigators may view the approach as draconian, one solution is to change locks on housing rooms prior to announcing quarantine. A meeting with personnel affected by the quarantine should be scheduled as quickly as possible. During that meeting, investigators and their staffs should be informed of the facts as known at the time and the proposed management plan. Access to rooms operating under quarantine conditions should be limited to the extent possible, preferably to a single individual from each lab (often the lab manager). Breeder cages should be broken down so pups will not be delivered after a certain point in the quarantine process. Litters that are within a few days of weaning may be retained. New animals, either from approved vendors or from other locations on the campus, should never enter a room quarantined for a virus infection, thus limiting the population of susceptible animals. If investigators housing mice in the affected room(s) have standing orders with approved vendors, those orders should be cancelled for at least 90 days, allowing time for an initial 100% screen of the room plus two additional screens at 21- to 28-day intervals. A critical early step is tracking any relocations or exports that have occurred within the last 90 days prior to detection of the excluded agent. If either has occurred, the destination room(s) must be tested for 'collateral damage' and/or recipient institutions must be notified that the animals received may have been infected. The scientist(s) having mice in the affected room might also house mice in other rooms and/or vivaria on the campus. Rodents in those areas should also be screened since traffic patterns of laboratory staff usually cannot be easily monitored. Supplies exiting a room operating under quarantine conditions should be bagged and autoclaved to reduce chances of further spread within the vivarium. This, of course, adds substantially to the workload of husbandry staff and may necessitate overtime and, thus, increased financial burden to the animal resource. Each cage in a quarantined room is sampled (e.g., serum for serology provided the principals are immunocompetent or feces for PCR to detect enterotropic pathogens) at 3- to 4-week intervals. Two consecutive negative screens of 100% of cages in the room are required to lift quarantine. Depending on the prevalence of infection at the baseline interval – and the nature of the mice – the management approach may be test-and-cull or the room may be depopulated by a combination of culling and rederivation (Smith, 2010). As discussed earlier, rederivation can be accomplished by a variety of means and the choice may hinge on the age and fecundity of the available animals. If the infecting agent is zoonotic, the institution may very well opt for depopulation via euthanasia, decontamination, and safe disposal.

Because pathogens often cause immunological perturbations, and because these disturbances can persist even in recovered animals (Compton *et al.*, 1993), the use of infected animals in immunological research should be avoided. Tissues, organs, fluids, or tumors from mice in a room quarantined for a virus infection should never be collected for transplant into other animals. A virus might contaminate these materials, especially if it causes a persistent infection (Compton *et al.*, 2004d; Riley *et al.*, 1960), has a broad host range (Bhatt *et al.*, 1986a), or has a predilection for replicating in rapidly dividing cells (Bonnard *et al.*, 1976; Mckisic *et al.*, 1993).

Control and eradication are most reliably achieved by depopulation, disinfection, and repopulation with SPF replacements or rederived descendants of the infected colony. Proper chemical disinfection following

depopulation is critical to preventing the recurrence and spread of an outbreak. Consequently, the principles of effective chemical disinfection, reviewed in Section III, will be restated here. Thorough sanitization and disinfection of the affected area are necessary to prevent recurrence or spread throughout a facility. Animal areas (including shared equipment, such as anesthesia equipment or behavioral testing apparatus) should be cleaned with detergent, then, at a minimum, disinfected with compounds known to inactivate the contaminating infectious agent. General cleaning should be undertaken before disinfection or sterilization, because chemical disinfectants are inactivated by the presence of organic matter (animal room soil). Detergents are best for removing soil, whereas disinfectants and sterilants work best on clean surfaces. Sterilants will, by default, kill a broader range of organisms, and therefore are usually the best choice for general decontamination purposes. Such sterilization agents include, but are not limited to, vapor-phase hydrogen peroxide, chlorine dioxide gas, and formaldehyde. A general plan, if a room has been contaminated, might consist of the following:

- Depopulate the room.
- Discard any nonessential or easily replaceable equipment.
- Place any materials leaving the room in a bag; spray the outer surface of the bag with a disinfectant and then place it into a second bag outside the room. The bagged caging may then be autoclaved, cleaned, and then autoclaved again before reentering a clean room.
- Clean the floors, walls, and room surfaces thoroughly with a detergent solution and then rinse.
- After rinsing, apply an aqueous-based disinfectant as per the manufacturer's recommendations (concentration, time, temperature, humidity, etc.).
- Rinse again.
- Apply at least one other aqueous-based disinfectant (with a different mode of action than the first). Many facilities will go further and use a sterilant such as vapor-phase hydrogen peroxide or chlorine dioxide.

When this approach is not feasible for scientific or financial reasons, a test-and-cull protocol can be implemented with recognition that this may be a protracted process. In all cases, steps should be taken to ensure that the likely sources of infection are adequately disinfected or eliminated. The postquarantine process should include encouraging investigators to cryopreserve unique genotypes. This ensures that unique strains will not be lost in future outbreaks or disasters such as floods or prolonged power outages. This strategy can have the added benefit of reducing per diems for on-the-shelf animals that are less frequently needed for experiments. It also conserves cage space, which is an advantage for the animal resource and colleagues who may need additional space.

C. Investigation

One of the first questions an animal resource representative may hear at the first user meeting scheduled after imposition of quarantine is: how did this happen? In many cases, that question remains unanswered. Nevertheless, systematically investigating the potential direct and indirect sources of contamination discussed in Section III often reveals procedural or engineering gaps that can be easily closed to reduce the chance that outbreaks will recur. A few of the possibilities are noncompliance with SOPs for animal importation and on-campus relocations; noncompliance with human traffic flow SOPs; improper disinfection of supplies; failure by animal resource to calibrate and maintain equipment; improper use of PPE; improper storage of diet or other supplies used by investigators; improper handling of cages by lab staff (opening cages outside a laminar flow hood); home maintenance of reptiles or rodents by staff members; and injection of biological products that have not been tested for adventitious agents. The advent of bar-coded cage cards has permitted much improved on-campus tracking of cages that have relocated. Vivaria should also have the capacity to store experimental diets so that investigators do not keep these in the lab which may have less rigorous pest control practices than the vivarial space.

In order to detect outbreaks earlier, institutions with ventilated racks might consider the use of PCR testing of swabs taken of exhaust air dust and of noninvasive fecal and swab specimens collected directly from resident and quarantined animals, and further mitigation of risks associated with possible sources of infection include autoclaving or gamma irradiation of feed and bedding. If significant noncompliance is shown to be responsible for an outbreak, mandatory retraining and/or Institutional Animal Care and Use Committee intervention may be imposed.

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