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Review

Livestock and Risk Group 4 Pathogens: Researching Zoonotic Threats to Public Health and Agriculture in Maximum Containment

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Abstract

Maximum-containment laboratories are a unique and essential component of the bioeconomy of the United States. These facilities play a critical role in the national infrastructure, supporting research on a select set of especially dangerous pathogens, as well as novel, emerging diseases. Understanding the ecology, biology, and pathology at the human-animal interface of zoonotic spillover events is fundamental to efficient control and elimination of disease. The use of animals as human surrogate models or as target-host models in research is an integral part of unraveling the interrelated components involved in these dynamic systems. These models can prove vitally important in determining both viral- and host-factors associated with virus transmission, providing invaluable information that can be developed into better risk mitigation strategies. In this article, we focus on the use of livestock in maximum-containment, biosafety level-4 agriculture (BSL-4Ag) research involving zoonotic, risk group 4 pathogens and we provide an overview of historical associated research and contributions. Livestock are most commonly used as target-host models in high-consequence, maximum-containment research and are routinely used to establish data to assist in risk assessments. This article highlights the importance of animal use, insights gained, and how this type of research is essential for protecting animal health, food security, and the agriculture economy, as well as human public health in the face of emerging zoonotic pathogens. The utilization of animal models in high-consequence pathogen research and continued expansion to include available species of agricultural importance is essential to deciphering the ecology of emerging and re-emerging infectious diseases, as well as for emergency response and mitigation preparedness.

Key words: Biohazard containment, BSL-4, Ebola virus, Hendra virus, livestock, Nipah virus, Reston virus, risk group-4 pathogens

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INTRODUCTION

The ecology of emerging and re-emerging viruses, including viral spillover and intra-species transmission, is complex, and the ability to decipher these intricacies often requires multi-disciplinary approaches and, not infrequently, scientific investigation involving the use of laboratory animals. Understanding the ecology of spillover events involving zoonotic pathogens at the human–animal interface is fundamental to efficient control and elimination of disease.[1] The use of animals as models in research is an integral part of unraveling the interrelated components involved in these dynamic systems, and these models can prove vitally important in determining the viral and host factors associated with virus transmission. Furthermore, determination of the susceptibility of animals representing species of interest can be critical to developing effective monitoring and intervention strategies. More than 60% of known human pathogens are zoonotic, with 75% of all emerging infectious diseases causing zoonoses.[2–6] Understanding disease dynamics of virus–host systems can further define the mechanisms of transmission and maintenance within that system, and the resulting information can be developed into better risk mitigation strategies.[4] As novel pathogens continue to cause major public health and economic concerns worldwide, the use of animals in infectious disease research will continue to be critical in determining the best means to predict, prevent, and control these diseases.

Historically, traditional animal infection models, such as rodents and non-human primates (NHPs), have filled a critical scientific need as a surrogate means of studying the pathogenesis of infectious diseases in humans. Varying aspects of a disease of interest may be simulated through the use of different models and scientific techniques, but this approach typically requires the understanding of key elements of the human disease they are meant to reflect. With advances in transgenic technologies, it has become possible to design fit-for-purpose animals, greatly expanding the utility of these tools in scientific endevors.[7] In the case of humanized mice, immunodeficient animals can be injected with human hematopoietic stem cells, resulting in mice with functional human cells, tissues, and organs.[8,9] Small animal models designed for pathogenesis studies have also allowed for the initial assessment of candidate human vaccines and therapeutics.[10] Well-developed small animal models tend to replicate the virus of interest, ideally inducing reproducible clinical disease and pathology that closely mimics human illness based on natural routes of exposure.[11] The use of domestic animals, including species of livestock, can complement small animal models yielding more rigorous findings.[12] Additionally, livestock use in translational medical research can also better reflect the complexity of an outbred species compared with inbred rodent strains.[13]

The utility of agricultural animals, such as pigs, cattle, sheep, and goats, in biomedical research is longstanding, with many groundbreaking studies attributed to their use.[14,15] Of these, pigs may be the most utilized model in translational research and have contributed to major scientific breakthroughs in gastrointestinal and pulmonary disease as well as xenotransplantation research.[12,16–19] With advancements of new technologies in gene editing, generating transgenic animals is becoming more accessible. For example, CRISPR technology has addressed many of the challenges of producing animal species with tailored genetic modifications, and the extension of this technology to livestock has greatly expanded their value to biomedical investigations.[7,20] These advancements, including applications as models ranging from cardiovascular diseases, cancer, and diabetes mellitus to cystic fibrosis and forms of muscular dystrophy, have been extensively reviewed elsewhere.[7,20–22] The utility of agricultural animals is projected to continue to expand as a means to supplement or improve other translational models of human medicine.[16] Agricultural animals are not solely used to recapitulate human disease; this review will describe the critical role livestock have played in advancing the understanding of disease ecology and in the protection of food security and public health.

Although animal models for infectious diseases are critical for understanding the associated pathogens, there are inherent disadvantages. Conducting in vivo studies with infectious pathogens, regardless of the biosafety level, substantially increases the associated biorisk compared with in vitro work.[23] Further, working with livestock adds additional challenges because they cannot be housed in individually ventilated caging and therefore require the housing room to serve the purpose of primary containment.[24] For personnel to work in this space, they have to physically enter this primary container, which is in opposition to maximum containment research involving traditional laboratory animals where the primary container (eg, cage) is typically transported into a biosafety cabinet or animal manipulation is performed on a downdraft table.[24] However, work with NHPs can be a common exception to this practice. The differences between the biosafety levels, as they relate to the pathogen classification, regulatory requirements, and terminology used in the United States, will be discussed throughout this article, with the focus on maximum containment, Biosafety Level 4 (BSL-4) facilities.

Biomedical research encompasses a variety of models, ranging from biological, including whole animal and ex vivo systems, to non-biological, such as mathematical and computer-based modeling. Whole animal models are traditionally thought of as surrogates for a human biologic system to differentiate our understanding between normal and abnormal function and provide insight into intervention or preventative measures.[25] Conversely, in this article, the animal systems being discussed often serve as experimental models to assess the involvement of animals belonging to the species of interest in the life cycle of a pathogen. This typically includes elucidating whether an animal can serve as a spillover, intermediate, and/or amplifying host. Alternatively, the animal in question may serve as a reservoir capable of either sustaining the pathogen in the environment or as a risk for spillover transmission to other susceptible animals, such as humans. In this case, these animals are considered models for what may occur during natural infection and serve as surrogates for members of their own species. These “target-host models” are models of species-specific animal disease and typically involve challenging target hosts by experimental exposure to a virus to determine susceptibility, understand transmission, and evaluate the potential risk of spillover into the human and/or production animal population. This research can serve as a sound basis for risk assessments and policy decisions to determine how to deploy limited resources during a time of need.[26] In this article, we exclude the historical use of large animals for reagent production for Risk Group 4 (RG4) pathogens. At this time, the authors are not aware of any agriculture animal serving directly as a surrogate model for research into RG4 human disease. This does not preclude the development of their use in the future, especially with the increased utilization of pigs in biomedical research and the historical use of animals of this species as surrogates for many human diseases at lower levels of containment. In fact, pigs have been the predominate agricul-

turally important animal used in BSL-4Ag research. Though this article will largely discuss domestic pigs, the contribution and use of other livestock will be mentioned.

Experimental infection models utilizing animals in maximum containment typically fall into 2 broad categories: (1) models for the evaluation of vaccines or antiviral treatment, typically as surrogates for application in humans; or (2) target-host models, those for use in studies designed to investigate viral replication, antibody development, viral shedding, transmission, and pathogenesis in naturally infected animals. Livestock utilized in high-consequence pathogen research usually fall into the latter category and are routinely used to establish data to assist in risk assessments. However, this article will touch on studies that have been conducted for development of preventative measures in livestock such as Hendra virus (HeV) in horses and Nipah virus (NiV) in pigs.[10,27] Although other commonly used laboratory animals (mice, hamsters, ferrets, NHPs, etc) have been routinely utilized to expand our knowledge of these pathogens and the human diseases that they cause, their contributions will not be the focus of this article because recent reviews are available elsewhere.[10,28,29] Here, the focus will be on the use of livestock (predominately horses and pigs) in maximum containment, BSL-4Ag research, and we will provide an overview of historical use involving RG4 pathogens in agriculture animals. Discussion on henipaviruses (HeV and NiV) and the filoviruses (predominately ebolaviruses) will highlight the importance of animal use, insights gained, and how this type of research is essential for protecting animal health, food security, the agriculture economy, and human public health in the face of emerging zoonotic pathogens.

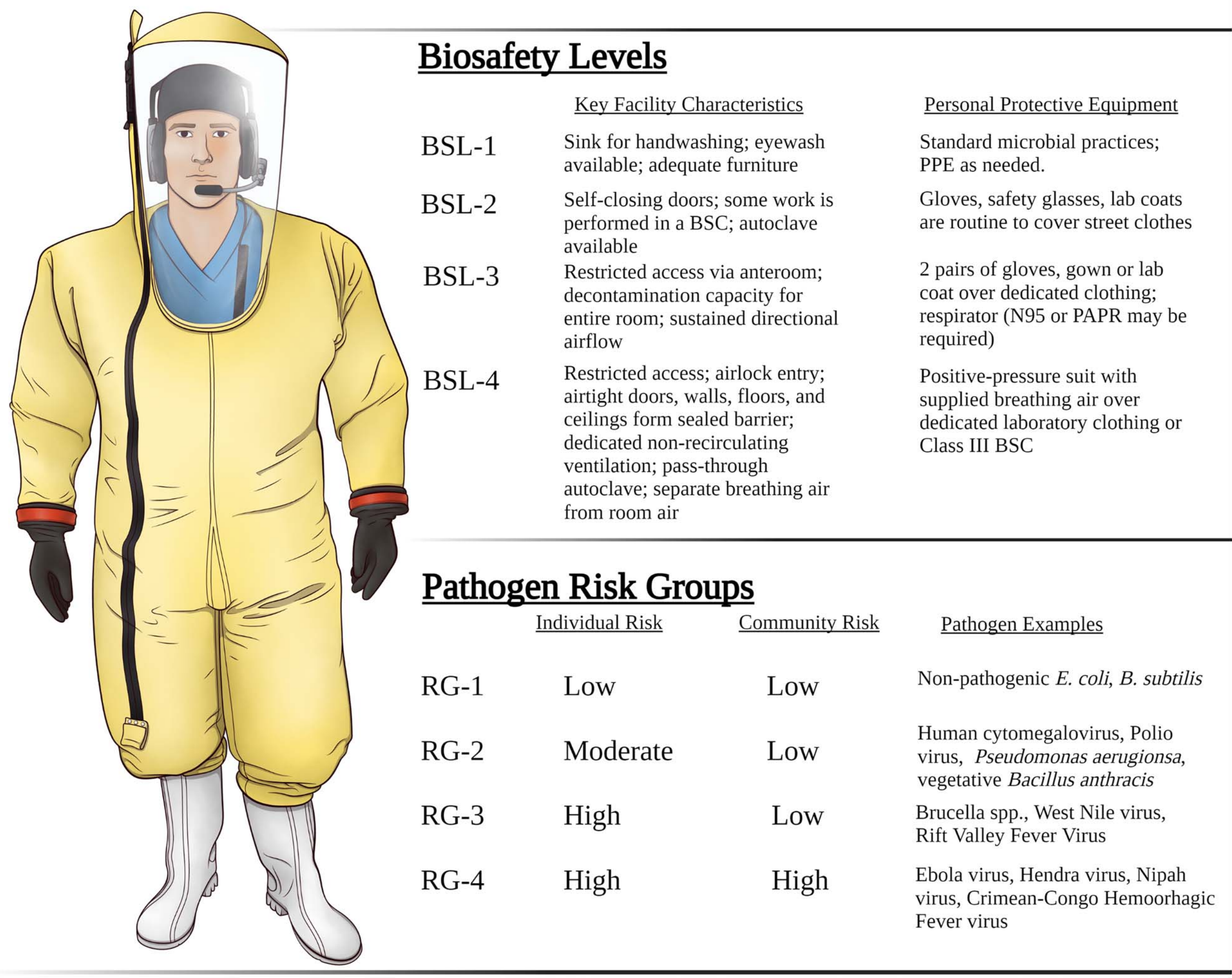
MAXIMUM CONTAINMENT FACILITIES, LIVESTOCK, AND RG4 PATHOGENS: CONSIDERATIONS AND CONSTRAINTS

The 4th edition of the World Health Organization's Laboratory Biosafety Manual defines biosafety as encompassing "containment principles, technologies and practices that are implemented to prevent unintentional exposure to biological agents or their inadvertent release."[30] Along with biosecurity programs, these components are incorporated through the development of a safety culture and risk assessment framework as elements that are fundamental to protecting both the laboratory workers and the wider community.[30] In the 6th edition of Biosafety in Microbiological and Biomedical Laboratories, the predominate focus of biosafety is on addressing facility engineering and personnel behavior, which includes the actions used to conduct the research; the overarching principles are containment and risk assessment.[24] Containment in this context refers to microbiological processes, safety equipment, and facility safeguards meant to protect workers, the environment, and the public from the pathogens being utilized. Risk assessment refers to the "…process that enables the appropriate selection of microbiological practices, safety equipment, and facility safeguards that can prevent laboratory-associated infections…."[24] Because the standards and requirements for biosafety vary by country, the focus of this article and the terminology used will be that considered standard in the United States, including biosafety levels and pathogen risk classification (Figure 1).

Biosafety levels as described in the 6th edition of Biosafety in Microbiological and Biomedical Laboratories range from the lowest level, BSL-1, to the most restrictive level requiring maximum containment, BSL-4. These levels can be further delineated as Animal Biosafety Levels (ABSL-), used to designate the use of traditional laboratory animals, and Agricultural Biosafety Levels used to describe the use of livestock (eg, BSL-4Ag) (Figure 1).[24] Risk Groups (RG) are utilized to describe the characteristics of the pathogen that can inform the determination of which biosafety level is required to conduct research on that pathogen.[31] RG are classifications that describe the hazard posed by infectious agents or toxins in the laboratory setting and are designated from 1 to 4, with a higher value representing increased risk. Ranking typically takes into consideration characteristics such as pathogenicity, severity of disease, mode of transmission, host range, community risk, and the availability of effective preventative measures or treatments, and these RG designations can be country specific depending on the regional risk assessment and endemicity (Figure 1).[31,32] For example, in the United States, Ebola virus is classified as an RG4 pathogen and requires a BSL-4 facility; therefore, research involving livestock as a model will typically be conducted in a BSL-4Ag facility.[24,30] However, RG levels do not always correspond to the same numbered biosafety level. RG4 agents are associated with serious or lethal human diseases for which preventative or therapeutic interventions are not usually available.[31] All RG4 pathogens are viruses, and those discussed here in association with livestock are bat associated (ebolaviruses and henipaviruses). Brake et al, also published in this themed issue, provide an extensive review of zoonotic RG3 and RG4 pathogens, including the benefits and challenges of developing associated veterinary vaccines.[33]

Many of the general, non–maximum containment–specific safety concerns with utilizing livestock in biomedical research are reviewed elsewhere.[15] A BSL-4 rating in the United States requires work to be conducted in 1 of 2 ways: within a "cabinet laboratory" where manipulation of pathogens is performed in a Class III biosafety cabinet (BSC) or in a "suit laboratory" designed for the use of positive-pressure suits with a dedicated breathing air supply.[34] Information in this review will focus on research performed in facilities utilizing suits, because cabinet laboratories do not facilitate work with livestock and have been described elsewhere.[35] Positive-pressure suits, as shown in Figure 1, are a self-encapsulating, full-body, protective barrier with an umbilical-fed external, HEPA-filtered air supply that provide the researcher protection by 2 main functions: pressure and physical barrier. The pressure provided by the in-line air helps ensure that air is expelled out of the suit and away from the wearer if a breach of suit integrity occurs. Secondly, the robust material used to construct the suit provides a physical barrier separating the researcher from the environment within the laboratory.[34,36] Researchers move around the containment space by switching between available air-line connection points. When exiting the BSL-4 laboratory, a chemical shower is used to decontaminate the outside surface of the suit, typically via a 2-stage process involving disinfectant spray followed by a rinse cycle with the primary objective being the removal of gross microbial contamination.[37,38] For general information concerning the safety considerations in BSL4 laboratories, see the detailed requirements and best practices outlined by Bressler and Hawley[35] and the special considerations for animal agriculture pathogen-specific biosafety as reviewed by Heckert, Kozlovac, and Balog.[39]

Hazards that are considered routine and easily mitigated in lower levels of containment can lead to serious consequences in BSL-4 conditions.[35] Routine activities in standard BSL-2 laboratories such as pipetting, making aliquots, injecting animals, and processing samples are more laborious in maximum



Biosafety Levels

	Key Facility Characteristics	Personal Protective Equipment
BSL-1	Sink for handwashing; eyewash available; adequate furniture	Standard microbial practices; PPE as needed.
BSL-2	Self-closing doors; some work is performed in a BSC; autoclave available	Gloves, safety glasses, lab coats are routine to cover street clothes
BSL-3	Restricted access via anteroom; decontamination capacity for entire room; sustained directional airflow	2 pairs of gloves, gown or lab coat over dedicated clothing; respirator (N95 or PAPR may be required)
BSL-4	Restricted access; airlock entry; airtight doors, walls, floors, and ceilings form sealed barrier; dedicated non-recirculating ventilation; pass-through autoclave; separate breathing air from room air	Positive-pressure suit with supplied breathing air over dedicated laboratory clothing or Class III BSC

Pathogen Risk Groups

	Individual Risk	Community Risk	Pathogen Examples
RG-1	Low	Low	Non-pathogenic *E. coli*, *B. subtilis*
RG-2	Moderate	Low	Human cytomegalovirus, Polio virus, *Pseudomonas aerugionsa*, vegetative *Bacillus anthracis*
RG-3	High	Low	Brucella spp., West Nile virus, Rift Valley Fever Virus
RG-4	High	High	Ebola virus, Hendra virus, Nipah virus, Crimean-Congo Hemoorhagic Fever virus

Figure 1: Overview of biosafety levels (BSL) and pathogen risk groups (RG). As described in *Biosafety in Microbiological and Biomedical Laboratories* (BMBL), 6th Edition, the essential elements of BSL are standard microbiological practices, safety equipment, and laboratories facilities.[24] The 4 levels are cumulative, building in ascending order on the required protection to personnel, the environment, and the community. (A) An example of a positive-pressure suit used in a BSL-4 research setting providing a self-encapsulating, full-body, protective barrier with an isolated, umbilical-fed breathing air supply. (B) The table shows the BSLs and examples of the associated key facility characteristics and the personal protective equipment. (C) The table shows pathogen risk groups demonstrating the risk level to the individual and the environment as well as examples of pathogens of each RG. BSC = biosafety cabinet; BSL = biosafety level; PAPR = powered air-purifying respirator; PPE = personal protective equipment; RG = risk group.

containment due to the safety considerations and use of positive-pressure suits. Peripheral vision, though improved with newer suit designs, is restricted, and communication between personnel is difficult due to the noise generated from the in-line air supply. An example of how increased safety measures complicate functionality is the reduction in dexterity through the use of additional layers of gloves. For instance, 2 layers of examination gloves are typically worn, with the layer closest to the skin taped to the cuff of long-sleeved gowns or scrubs. A third layer of hand protection is provided by abrasion- and chemical-resistant gloves attached to the suit (18-mm-thick neoprene gloves are utilized at the authors' facility). Another layer of examination gloves are usually worn over the suit glove and donned/doffed when changing activities in the laboratory. During injection, sampling, and necropsy of animals, it is not unusual for a sharps-resistant glove and an outer examination glove to be worn on the non-dominant hand, external to the suit gloves. In the last scenario, a total of 6 layers of gloves may be worn, all providing increased levels of protection yet also resulting in increased restriction to hand and finger dexterity. A local risk assessment should be carried out by the institution to determine the most appropriate style, material, and number of gloves required for the task to ensure the safest practice will be followed.

BSL-4Ag facilities typically involve working with livestock maintained in loose-housed or an open-pen setting (Figure 2).[24] Working with large animals while wearing a positive-pressure suit in this manner can be challenging, because penning and gating required for housing can compromise the protective suit through crush, pinch, and puncture points.[39] Because some livestock animals tend to investigate their surroundings by chewing, maintaining suit integrity while in an animal pen is a constant concern yet paramount to personnel safety. This can be especially challenging with animals such as domestic pigs that are naturally inquisitive and like to root and chew on the boots or suit legs, which can easily lead to punctures and tears in

the material. When manually restraining livestock, their hooves or claws can also lead to punctures of the personal protective equipment. For these reasons, many additional protective measures must be in place to maintain positive-pressure suit integrity such as the use of bite-resistant gloves, use of chemical sedation in place of physical restraint, and modification of penning to reduce "pinch points." Alternatively, the use of a squeeze gate or board with injectable sedation can help to prevent physical contact; however, the administration of a sedative requires sharps (syringe and needle), and therefore a local risk assessment must be performed to determine the best practice.

The heightened levels of regulatory oversight, both in occupational health and safety as well as biosecurity, and the restrictive nature of performing research in a BSL-4 facility, combined with highly specialized and logistically challenging maintenance requirements, make maximum containment facilities very expensive to operate. Due to this expense, these facilities are typically government owned and operated or heavily government subsidized.[33,40] It is estimated that the annual operating costs of a facility are roughly 10% of the construction costs.[33,40,41] Development of adequate staffing adds to the difficulties in facility operations because the workforce must be highly qualified and are typically uniquely specialized, leading to multi-disciplinary teams requiring intensive and continuous training.[24,30,40,42] Due to the expense of building and maintaining these facilities, there are currently only 4 BSL4-Ag–rated facilities in the world, with a fifth near completion (Figure 3). All of these facilities are government owned and operated and include the Commonwealth Scientific and Industrial Research Organization's Australian Centre for Disease Preparedness in Geelong, Victoria, Australia (previously known as the Australian Animal Health Laboratory [AAHL]); the Canadian Food Inspection Agency's National Centre for Foreign Animal Diseases (NCFAD) in Winnipeg, Manitoba, Canada; the Federal Ministry for Food and Agriculture's Friedrich-Loeffler-Institut in Insel Riems, Greifswald, Germany; and the Chinese Academy of Agricultural Sciences' Harbin Veterinary Research Institute in Harbin, Heilongjiang, China.[33,41] The United States Department of Agriculture's National Bio- and Agro-defense Facility (NBAF) is near completion in Manhattan, Kansas, USA, with an expected construction completion date of late 2021 and being fully operational by late 2024. Similar to the other laboratories listed, the NBAF will have BSL-3, BSL-3Ag, BSL-4, ABSL-4, and BSL-4Ag capabilities. The limited availability of BSL-4Ag facilities specifically designed to house livestock and the dire need to progress scientific knowledge with these animals has encouraged research to be performed in livestock using modified ABSL-4 facilities, including the use adapted handling protocols and limiting animal size, and through the use of creative housing options, such as soft-sided containment enclosures.[33,43]

STUDY DESIGN CONSIDERATIONS FOR LIVESTOCK ANIMAL MODELS USED IN MAXIMUM CONTAINMENT

Wood and Hart state that "arguably the single most essential element in animal-based research, identifying and selecting the most appropriate animal model is also the most challenging."[44] Animal models are critical to the effective study of infectious diseases and, after determining that appropriate non-animal alternatives are not available, these models are needed to fulfill Koch's postulates. As with traditional laboratory animals, there are numerous factors that must be considered for the identification and development of livestock as a model. Selection criteria have been extensively reviewed elsewhere[11,44,45] along with Institutional Animal Care and Use Committee considerations.[46–48] Beyond this, there are additional, specific concerns when developing large animal models for research in a BSL4-Ag setting.

When contemplating the use of livestock in RG4 pathogen research, one of the primary concerns must always be the safety of the personnel that will be conducting the research, and this is especially true in regards to how the animals being considered can be handled in the facility. To mitigate some safety concerns, many programs typically require animals to be sedated anytime they are to be manipulated, even for otherwise routine tasks. For instance, at our facility, animals inoculated with RG4 pathogens are sedated for routine blood collection, because this not only makes it easier to examine and position the animal while wearing a positive-pressure suit but also reduces the likelihood of a needle-stick injury to the person collecting the sample as well as the person restraining the animal. Sedatives may be administered with minimal, short-term physical restraint (for instance, physically restraining an animal while masking with isoflurane) or using a syringe pole to administer an injectable agent. Extreme caution coupled with hazard mitigation measures must be observed anytime sharps (needles, scalpels, etc) are utilized in a BSL-4 facility.

One consideration for model development is the challenge virus route and dose used to expose the animal to the pathogen of interest. For instance, if the goal is to mimic natural infection, oronasal inoculation may be preferred over venous or peripheral injection. The amount of pathogen given to the animal is also a concern because "over-challenge" may lead to development of a disease when the dose is beyond that reasonable for natural exposure. It has also been proposed that over-challenge may also lead to masking the natural susceptibility of animals of interest, because a more robust immune response could prevent disease from occurring, therefore minimizing the perceived risk.[43,49] Consideration of social housing of livestock in a maximum containment setting is another critical concern to the study design. The benefits of social housing include both improved animal welfare as well as increased scientific validity, because singly housing social animals may mask the development of pathogen-specific, abnormal behavior. For instance, singly housing social animals such as domestic pigs can lead to extreme depression as a physical manifestation in that animal. However, beyond the facility constraints discussed by others,[50] group housing presents the additional challenge of limiting the recognition of clinical signs because it can be difficult to single out 1 animal of a herd while making clinical observations. Social housing should be considered an animal welfare, facility, and study design concern, but when possible, every effort should be made to allow for group housing to occur.

As with all animal studies, there are conflicting goals of ensuring scientific validity by using enough animals to reach statistical significance and also limiting the number of animals used. The number statistically required for a power analysis may not always be feasible, or even ethical, with livestock in biocontainment conditions. Further, the amount of BSL4-Ag animal housing space available will dictate the number of animals, age, and size, which are invariably influenced by the type of animal utilized. During study design, the number of animals to use is also influenced by the overall study goals. For example, the personnel performing necropsies of a RG4 pathogen-infected animal are inside the containment zone, creating additional

	ABSL-4	BSL-4Ag
Similarities:	Unique engineering and design features to prevent microorganisms from dissemination into the environment and to protect personnel working in the facility. Redundancies in life support and facility control systems. Strict access control limits those who can enter the facility.	
Facility types:	1. Positive-pressure suit facility 2. Class III cabinet facility	1. Positive-pressure suit facility is the only option
Examples of animals used:	Mice, hamsters, ferrets, non-human primates	Pigs, cows, horses
Primary biocontainment for animals:	IVC or caging housed in flexible film isolators serve as the primary biocontainment barrier for infected animals. Most infected animals are handled in a primary barrier system. For example, infected mice housed in IVC and transported to a Class II BSC for manipulation.	Animals are kept loose-housed or in open-penning and the rooms serves as the primary biocontainment barrier. Livestock are typically too large to house in isolators following experimental infection.
Necropsy:	Necropsies are typically performed in a BSC or on a downdraft table.	Necropsies are performed in the room as livestock will not typically fit inside a BSC or on a downdraft table.
Additional measures and/or concerns:		Livestock typically require additional animal restraint devices, as well as penning and gating, that can serve as hazards for personnel. Movement of animals and carcasses due to their size is more challenging.

Figure 2: Characteristics of animal biosafety level-4 (ABSL-4) and biosafety level-4 agriculture (BSL-4Ag) facilities and operations. There are many shared characteristics between ABSL-4 and BSL-4Ag facilities, including the measures taken to protect personnel working in the space and to protect the environment and general population from the pathogens being researched. A prominent difference between these BSL-4 enhancements is the animals being utilized and the options for housing those animals. Because livestock are typically too large to fit in individually ventilated cages or flexible film isolators, they must be housed loose or penned in the room. For a more detailed description of BSL-4, ABSL-4, and BSL4-ag, see section V and Appendix D of the 6th Edition of *Biosafety in Microbiological and Biomedical Laboratories* (BMBL). IVC = individually ventilated caging; BSC = biological safety cabinet.

risks that must be mitigated. Necropsies in a BSL4 setting must be performed in a slow and methodical manner, paying careful attention to prevention of gross contamination of the suit and gloves as well as adhering to strict safety protocols for using sharps. Doing this is time consuming, and the number of necropsies that can be performed in a given day is limited compared with lower levels of containment. Studies that require detailed necropsies and extensive post-mortem sample collection must take this into consideration when determining animal numbers in the study and timing of inoculations. The acclimation period of animals must also be considered during study design because there are significant differences in the BSL4-Ag environment compared with other housing (increased room air exchanges, loud noises from the opening and closing of pneumatic-seal doors, interactions with personnel wearing positive-pressure suits, and constant negative room pressure). Large animals, such as horses and ponies, should be temperament tested prior to being included in a study.

As mentioned, BSL4-Ag facilities are extremely expensive to operate, and these expenses exponentially increase with the size and addition of animal housing rooms. Therefore, facility constraints also impact model selection by influencing the range of livestock of various species that can be used, as well as the number, breed, and age of animals included in studies. For instance, ponies may be used in place of horses and miniature breeds may be preferred. Young pigs may prove easier to handle if physical restraint is required, and the smaller size may allow more animals to be accommodated in the housing space. These

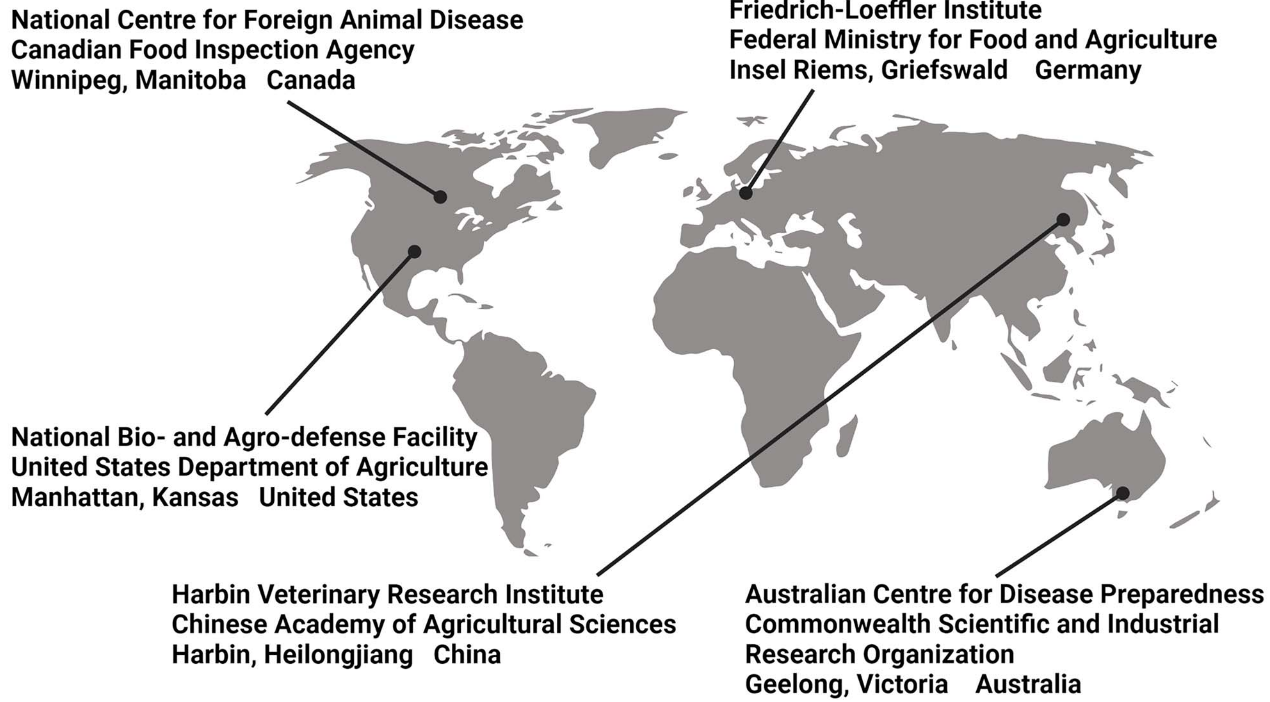


Figure 3: Locations and affiliation of the current biosafety level-4 agriculture (BSL-4Ag) facilities around the world. There are currently 4 operational BSL4Ag-rated facilities located in Australia, Canada, China, and Germany. A fifth facility is under construction in the United States, with a projected construction completion date of late 2021 and projected operational date of late 2024.

study decisions must be made in light of the potential biological influences they may have on the study outcome. As will be discussed with ebolaviruses, the age of pigs may affect the development of disease, even within a few weeks.[43,51] Ultimately, the model that should be selected is the one that, within research, safety, and facility constraints, best addresses the specific aim of the study.[11]

SUMMARY OF FACTORS INFLUENCING THE INTERPRETATION OF CHALLENGE STUDIES INVOLVING RG4 PATHOGENS

Below is a summary of some of the key factors the authors' consider as influencing the interpretation of studies involving livestock and RG4 pathogens:

- Challenge dose: "over-challenge" vs "under-challenge";[43,49,52]
- Route of inoculation/exposure: mimicking natural routes of infection, oronasal, use of a vectors for inoculation vs intradermal injection, aerosolization studies, etc;[10,53]
- Strain or isolate of challenge organism and potential for mutations to alter outcome: wild-type vs animal-adapted viruses;[54]
- In vitro passage of challenge virus and the potential for attenuation;
- Age, breed, sex, and genetics of the study animals;[43]
- Co-morbidities/co-infection of study animals with other pathogens;[55]
- Housing characteristics, including animal density, air flow, contact between animals, cleaning procedures, feed, etc; and
- Animal manipulations, including sedation, anesthesia, analgesia, etc.

These factors are important to consider in any challenge study but are especially important in maximum containment given the limited ability and costs, in monetary terms as well as in the use of live animals, to conduct these studies and the difficulty in conducting additional clarifying experiments.

HISTORICAL EXAMPLES OF THE USE OF LIVESTOCK IN RG4 RESEARCH

Henipaviruses

HeV and NiV are highly virulent, enveloped viruses with a negative-sense, single-stranded RNA genome within the *Henipavirus* genus, family *Paramyxoviridae*.[56] These viruses are considered unusual with respect to other paramyxoviruses due to their host promiscuity because they are capable of infecting animals of numerous species spanning 6 mammalian orders, including humans. Both viruses emerged during the last decade of the twentieth century and cause severe disease in humans, horses, and domestic pigs.[57] Pteropodid bats, including members of several species, are considered natural hosts of both HeV and NiV. As a reservoir host, bats become infected and seroconvert; however, active disease does not appear to occur.[56,58]

The Emergence of HeV. In September 1994, HeV emerged in dramatic fashion within the Hendra suburb of Brisbane, Australia, ultimately leading to the death of 14 horses by acute respiratory distress.[59] This outbreak was initially thought to be due to African horse sickness, a transboundary disease caused by an orbivirus exotic to Australia, until the subsequent death of a human in close contact. The outbreak also included the nonfatal infection of 7 horses and a second human case. Initially

labeled as equine morbillivirus, the causative agent was later recognized to be a novel pathogen that was named after the location it was discovered, Hendra. HeV became the first member of what is now the genus *Henipavirus*.[59] Although this outbreak represents the discovery of HeV, retrospective investigation determined that a fatal human case occurred 2 months prior and some 1000 km away from Brisbane. This case was found to be associated with the necropsies of 2 horses later shown to have died of the virus.[60,61] The infected human initially recovered and then relapsed 13 months later with severe encephalitis.[62]

Sporadic outbreaks of HeV occurred between 1994 and 2010, including 14 events transpiring in Queensland or the northeastern corner of New South Wales, Australia. Alarmingly, there were 26 events that occurred between 2011 and 2012, leading to heightened scientific and public concerns of HeV representing an unmanaged emerging zoonotic disease.[63] These concerns accelerated research leading to the development of a vaccine, the first commercially available product for protection against a RG4 pathogen for any animal, including humans.[27] To date, HeV is known to be responsible for disease in at least 7 human cases, including 4 that were fatal, demonstrating a case fatality rate of 57%, all with a history of close contact exposure to infected horses; yet the HeV attack rate for human exposure from infected horses is estimated to be 10%, a relatively low number.[64] There have been 106 documented cases in horses since 1994, representing 64 independent, natural spillover events. The most recent case occurred in July, 2021, in Queensland, Australia, and included the detection of a novel variant of HeV from a horse after acute fatal disease.[65,66] The case fatality rate for HeV in horses is estimated to be approximately 75%.[67,68]

HeV disease in horses is dramatic and can progress rapidly, with death possible within 3 days from the onset of clinical signs. Death is typically preceded by the development of acute febrile illness (up to 41°C rectal temperature, approximately 106°F), with frothy, blood-tinged, nasal secretions, increased respiratory effort (including tachypnea and dyspnea), tachycardia with rates reaching 120 beats per minute, and profuse sweating. Affected horses can also present as anorexic, depressed, and neurologic. Neurologic compromise typically includes head pressing and ataxia or presenting as laterally recumbent, with death typically following progression to convulsions and seizure-like activity.

The horse plays a central role in HeV transmission to humans because horses are presumed to serve as a spillover host from the bat reservoir. The majority of outbreaks in horses involve a single animal, suggesting that transmission between horses does not readily occur, and it is proposed that outbreaks involving multiple animals have been secondary to contamination and fomite transmission.[63,65] Domestic horses are exquisitely sensitive to HeV infection and appear to serve as an amplifying host in which the viral load reaches a threshold capable of facilitating transmission to humans. There have been no known human cases secondary to exposure to bats, and transmission in humans is thought to occur through direct exposure to secretions and tissues of infected horses either during clinical care, euthanasia, or post-mortem evaluation. No known human-to-human transmission has been documented.[69] Humans are, therefore, considered a dead-end host for HeV. The first known human case occurred in an equine trainer with death following development of severe interstitial pneumonia with subsequent acute respiratory distress.[70]

Animals representing multiple species have been shown to be experimentally susceptible to HeV infection with subsequent development of disease. Immediately following the first outbreak, studies were conducted to ascertain if a number of domestic animals were susceptible and to determine if an animal model could be developed that was more easily handled in biocontainment compared with horses.[70] Westbury and colleagues demonstrated that domestic cats developed disease comparable with horses when parenterally inoculated with HeV, including the development of a severe, fatal pneumonia. Guinea pigs were mildly susceptible, and mice, rats, rabbits, and chickens were not susceptible to infection. Two domestic dogs were also inoculated and although both remained asymptomatic, one animal developed antibodies. Though cats are readily susceptible to experimental infection, there is no evidence of natural infection with the virus or a potential transmission risk, even after an extensive serological survey of 500 cats from the Brisbane area.[70]

In a later, more extensive study, dogs were shown to be readily infected with HeV and able to shed live virus in oral secretions, and possibly urine, while remaining asymptomatic and developing virus-neutralizing antibodies.[71] Their susceptibility has been confirmed by the finding of at least 2 dogs being naturally infected; 1 was found to be seropositive with no signs of disease, and a second was found to be positive with recoverable virus after exposure to the blood of an infected horse.[71,72] Naturally, the capability of dogs to function in the transmission cycle is of great concern due to their close relationship with people.[71]

HeV and Horses: Understanding Advanced Through Laboratory Animal Research. The direct involvement of horses in the transmission of HeV made them a critical component and focus for intensive scientific investigation as a laboratory animal. The first animal experiments with HeV were conducted following the original outbreak, with the goal of reproducing the clinical syndrome observed in horses. This work was carried out at the Australian Centre for Disease Preparedness in Geelong, Victoria, Australia, and these experimental inoculations were successful at reproducing a similar clinical presentation to that observed in the field, leading to respiratory symptomology consisting of pulmonary edema and congestion as well as pulmonary hemorrhage. The virus was successfully isolated from the lung, kidney, and lymph nodes of the inoculated animals, thereby fulfilling Koch's postulates and confirming that HeV was the causative agent of the field cases.[70] Subsequent equine studies have demonstrated the presence of viral antigen in endothelial cells with systemic organ involvement, including the lungs, lymph nodes, kidneys, spleen, and gall bladder, as well as the central nervous system involvement evidenced by detection of viral antigen in the meninges.[73]

Protection of Horses and People: Development of the First Licensed Vaccine for an RG4 Pathogen. As a consequence of the drastic increase in the number of recognized HeV-related incidents occurring in Australia in 2011–2012, public outcry, including from the racehorse industry, pushed for government and scientific measures to control this zoonotic disease.[27,63] Supported by the findings of experimental inoculation studies in horses and in accordance with epidemiologic findings during outbreaks, it was concluded that horses in the immediate pre-symptomatic or symptomatic phase of HeV infection posed the most significant risk for transmission to humans. As shown by the increased risk to veterinarians, symptomatic horses come to the attention of medical caregivers that initiate clinical investigations and medical interventions that further facilitate human exposure to the virus.[27] With the politicized focus on the role of bats as carriers of the virus, the mass eradication of bat

populations was even proposed as a mitigation measure despite the potentially catastrophic environmental impacts.[74]

The actual mechanism that supports spillover transmission from bat populations to horses is not understood, but most likely reflects complex factors dependent on socioeconomic, environmental, and ecologic components.[5,27] Without advanced understanding of this process, there were no means of direct mitigation measures for breaking the cycle. It was not reasonable to eradicate the bat population, nor did there exist measures to prevent all interactions along the interface between bats and horses or horses and humans. Therefore, it was determined that the most direct approach at reducing the risk posed to humans would be suppression of virus replication in horses through the development and evaluation of an equine vaccine. A government, scientific, and private industry collaboration involved performing vaccine efficacy studies, challenging vaccinated horses with live HeV to determine the vaccines ability to prevent disease, and, critically, determining if vaccinated animals shed live virus, ultimately led to the availability of the first licensed and commercially available vaccine against an RG4 pathogen: Equivac HeV (Zoetis).[27] This vaccine is reviewed further by Brake et al in this themed issue.[33]

HeV and Pigs. After pigs were discovered to be involved in the transmission cycle of NiV, a virus closely related to HeV, there were concerns that domestic pigs could also be a transmission risk for HeV. Therefore, a study was conducted by Li et al at the NCFAD to determine the susceptibility and shedding potential of experimentally infected pigs as a means to inform public health risk assessments.[75] Through high-dose, oronasal inoculation of 4-week-old Landrace pigs and 5-month-old Gottingen minipigs, it was demonstrated that pigs are not only susceptible to HeV infection, but they are also capable of shedding live virus through oral, nasal, and rectal excretions, and, similar to the pathogenesis in horses, the virus was found to have an increased tropism for the respiratory tissues.[75] A serological survey conducted soon after the emergence of HeV surveyed 100 pig herds in Queensland, Australia, sampling a total of 500 animals and ultimately detecing no anti-HeV antibodies.[76] Li et al postulated that the lack of detection could be due to the incorporation of biosecurity measures at the surveyed piggeries but suggested that susceptibility of domestic pigs should heighten concerns of the potential role feral pigs may play, because the wild pig population is estimated in excess of 24 million in Australia.[75,77]

The Emergence of NiV. An outbreak of severe febrile illness in humans occurred in the fall of 1998 in Peninsular Malaysia, and, by the following spring, cases were detected in neighboring Singapore.[78–81] Initially considered to be caused by Japanese encephalitis virus, it was later realized that a novel paramyxovirus closely related to HeV was the causative agent.[78–81] Epidemiologic investigation demonstrated that infected individuals had close contact with domestic pigs, suggesting pigs may have a role in amplifying the novel pathogen. The newly discovered virus was named Nipah virus (NiV), and this initial emergence ultimately resulted in the culling of over 1 million animals and led to more than 265 human cases, of which 105 were fatal.[81] Emergency response measures halted the outbreak, but the required mass culling devastated the Malaysian swine industry. Following this initial event, no subsequent outbreaks of NiV have occurred involving domestic pigs.

Following the first documented outbreak of NiV in Malaysia, near annual events have occurred in Bangladesh and India.[82–91] Pteropodid bats, the primary reservoir of NiV, lead to sporadic outbreaks from direct spillover transmission from the reservoir host to humans through the consumption of date palm sap contaminated with excreta from bats.[89,92–94] Importantly, unlike the initial outbreak in Malaysia, these subsequent events identified human-to-human transmission, with a high case fatality rate typically above 70%.[95] Concerns over the potential for sustained human transmission of NiV has led the World Health Organization to add it to a list of priority diseases that pose the greatest public health risk due to epidemic potential and lack of available countermeasures.[96] In 2018, an outbreak of severe encephalitis caused by NiV resulted in a 91% case fatality rate in Kerala, India.[97] It is believed to be the result of spillover from bats; however, there is currently no definitive evidence to support this.[97,98] Interestingly, date palm sap is not cultivated in Kerala, suggesting spillover occurred through some other means, such as contaminated fruit.[92,99]

NiV and Pigs. Domestic pigs remain important to our understanding of NiV due to their role as a source of human infection during the 1998–1999 outbreak in Malaysia and their ability to recapitulate some aspects of human disease.[57,100] The initial detection and identification of NiV as the etiological agent was challenging, and early diagnostic detection remains a concern due the possibility of accidental or intentional release. This positions NiV as an agent of concern for potential use in a bio- or agroterrorism event. The low mortality, yet high morbidity rates in domestic pigs coupled with generalized, non-specific clinical signs raise concerns for timely recognition and diagnosis from an agriculture perspective as well. The infection rate in pigs during the 1998–1999 outbreak was estimated to be close to 100%; however, only approximately 1$–5% of animals succumb to disease.[81] Further, the age of pigs appeared to play a significant role during infection. Boars and sows presented with similar clinical signs, including an acute febrile illness accompanied by labored breathing, increased salivation, nasal discharge, and early-term abortion, or, rarely, sudden death.[81] In contrast, suckling pigs appeared to have a case fatality rate close to 40%, with a clinical presentation including open-mouthed breathing and paresis with muscle fasiculations.[81] The main driver of spillover transmission from pigs to humans during the outbreak was proposed to be secondary to droplet formation and spread from coughing pigs to humans in close proximity.[57,81]

Results of experimental infections of pigs with NiV vary based on the age of animal used and virus inoculum, including the isolate, dose, and route chosen. However, general observations tend to include the development of mild clinical signs such as fever, increased respiratory rate, and, less often, neurological involvement. Even though experimentally inoculated animals do not typically exhibit respiratory compromise, there is an observed pulmonary tropism of the virus leading to severe lung pathology with high viral loads detected by real time polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC), with subsequent isolation of live virus from a variety of tissues associated with the respiratory system.[101] A small proportion of infected animals develop severe neurological disease, including depression, unbalanced gait, muscle weakness, and head pressing, that typically progresses to humane endpoints with subsequent euthanasia.[102] Experimental pathogenesis studies with 5-week-old piglets have suggested central nervous system (CNS) involvement occurs by way of cranial nerves traversing the olfactory and trigeminal pathways.[102] Further studies demonstrated that pigs inoculated with a high dose of NiV

can result in exacerbated disease due to secondary bacterial infections, shown by the isolation of *Enterococcus faecalis* and *Staphylococcus hyicus* from skin lesions and findings suggestive of bacterial invasion of the CNS evidenced by the presence of turbid cerebrospinal fluid in multiple experimental animals.[103] The meningeal vasculature of 1 animal was congested and, on histopathology evaluation, was consistent with suppurative meningoencephalitis. The authors proposed that the typical interferon-based antiviral response was directly impaired by NiV infection, resulting in host immunosuppression leading to an opportunistic secondary bacterial infection.[103] These secondary infections could confound clinical investigations and lead to delays in recognition and accurate diagnosis of NiV infection in domestic pigs.

Experimental pig infection studies performed by Weingartl et al demonstrated clearance of NiV from most tissues by 23 days post infection (dpi), with only low amounts of viral RNA detected in the submandibular and bronchial lymph nodes as well as the olfactory bulb.[103] Viremia with NiV is shown to last substantially longer, with live virus isolated from the serum of infected pigs at 24 dpi.[103] It was shown that peripheral blood mononuclear cells are susceptible to NiV infection and that CD8+ lymphocytes were permissive to infection, and a significant lymphopenia was observed at 2 dpi in infected pigs compared with control animals. Comparing survivors with pigs with fatal outcomes, it was shown that a rebound of lymphocytes at 6 dpi is suggestive of recovery and eventual clearance of infection.[101] Stachowiak et al found that CD3 + CD6 + CD8+ T-lymphocytes are productively infected by NiV and contribute to viremia. The authors postulated that CD6 may contribute to NiV dissemination and targeting of small blood vessels, through its strong interaction with the ligand CD166 (also known as the activated leukocyte cell adhesion molecule) that is expressed at the microvascular endothelium of the blood brain barrier and upregulated through inflammation.[101]

The Malaysian (NiV-M) strain is the only one to have been implicated in an outbreak in domestic pigs. The finding of antibodies specific to the Bangladesh strain of NiV (NiV-B) in domestic pigs is suggestive of exposure, yet live virus has not been recovered from naturally infected pigs to confirm these findings.[104] To better understand the susceptibility of pigs to NiV-B, Kasloff et al performed an experimental inoculation study using a recombinant virus based on the genome of NiV-B.[105,106] Following oronasal inoculation, animals shed virus while generating dissemination throughout the animal comparable with that observed with NiV-M; however, animals remained asymptomatic. Unlike NiV-M, recombinant NiV-B viral RNA could not be detected in the spleen of animals and, on histological examination, pathologic differences could not be identified relative to controls. The authors noted that invasion of the CNS with NiV-B appears to occur by way of migrating infected monocytes and lymphocytes as opposed to invasion using the cranial nerves as seen with NiV-M.[105] Sera collected from experimentally infected animals displayed lower neutralizing activity against NiV-B compared with previous experimental exposure studies with NiV-M. In contrast to observations in a ferret model of infection, cross-neutralization of NiV-M and NiV-B by pig serum was not observed.[105,107] An important observation noted in the recombinant NiV-B exposure study was the finding of quantitative RT-PCR–positive rectal swabs possibly indicating a difference in intra-host tropism and presenting a possible non-invasive tool for screening suspect herds.[105]

Vaccine efficacy studies have been the predominant driver for experimental use of domestic pigs in NiV research, leading to expanded understanding of infection characteristics, including pathogenesis and mechanisms of host immunomodulation. A canarypox virus–vectored vaccine platform expressing the F and/or G proteins of NiV appears to be efficacious, yet there is currently no vaccine licensed for use in pigs.[108] A study evaluating the use of soluble G protein of HeV or NiV as an antigen for vaccination found that protection against NiV has shown promise as an efficacious means of conveying protection, yet these products have not progressed past the proof-of-concept stage of development.[109] Additional studies will provide valuable insight into correlates of protection against NiV infection and will, hopefully, progress to an effective vaccine for use in pigs for the protection of human public health and food security as well as animal health and agriculture-based economy.

NiV and Horses. The primary receptor for NiV, Ephrin-B2, is ubiquitous across many animals representing a diversity of species and is proposed to confer an unusually broad range of animals susceptible to infection.[110] Outbreaks have largely been associated with humans as the predominately affected animal, notwithstanding the initial outbreak of NiV-M in domestic pigs. However, broad serology-based surveillance studies have suggested the susceptibility of a broad range of animals.[104] Antibodies against the NiV glycoprotein were detected in cattle, goats, and pigs, but these sera were unable to neutralize NiV in vitro.[111] Moreover, dogs, cats, horses, and goats were shown to be serologically positive in infected areas during the Malaysian outbreak.[112]

Unlike HeV, NiV has not been implicated in large outbreaks in equine. However, in 2014, the Philippines experienced an outbreak of a NiV encephalitis-like disease in humans, and epidemiologic investigation found an increased risk associated with the consumption of horse meat.[113] The death of 10 horses from 2 villages occurred between March and May of that year with all but 1 of the animals displaying neurologic signs, including rapid progression of disease featuring head tilt, circling, and ataxia.[113] Epidemiologic field investigations found 17 individuals matching the human case definition with an extremely high case fatality of rate of 82%. Because not all human cases had a history involving the consumption or processing of meat, spillover directly from horses followed by human-to-human transmission was suspected.[113] Serological evidence identified neutralizing antibody titers against NiV, while next-generation sequencing characterized a 71-base pair fragment from the P gene aligning most closely to that of NiV-M. These results indicate that a NiV-like virus was responsible for infection of the horses leading to spillover and subsequent human-to-human transmission and outbreak in the human population.[113]

Ebolaviruses

Ebolaviruses are negative-sense, single-stranded RNA viruses that are members of the genus *Ebolavirus* in the family *Filoviridae* of the order *Mononegavirales*. This genus is subdivided into 6 species—*Zaire ebolavirus* (Ebola virus, EBOV), *Sudan ebolavirus* (Sudan virus), *Bundibugyo ebolavirus* (Bundibugyo virus), *Reston ebolavirus* (Reston virus, RESTV), and *Tai Forest ebolavirus* (Taï Forest virus)—with a recently added member of the genus, *Bombali ebolavirus* (Bombali virus). The *Filoviridae* family also includes the *Marburgvirus* genus, another group of lethal human pathogens, as well as numerous pathogens of other animals, including fish.[114,115]

Ebolaviruses are routinely associated with fascinating, scientifically perplexing, and sometimes terrifying accounts of unexpected emergence leading to outbreaks in humans with case fatality rates averaging approximately 40%, but have historically ranged from 0% to almost 90%, depending on the virus and the availability of medical intervention.[116] These viruses have captivated the general public and the scientific community as the basis of various books and movies, including Richard Preston's best seller, "The Hot Zone" (1994). This book was loosely based on the factual emergence of Reston virus in cynomolgus macaques (*Macca fasicularis*) in a quarantine facility in Reston, Virginia, in October, 1989, after transport of NHPs from the Philippines.

In July 2008, diagnostic assistance was requested from the US Department of Agriculture's Foreign Animal Disease Diagnostic Laboratory (FADDL) at Plum Island Animal Disease Center by the Philippine Department of Agriculture to help determine the etiologic agent of simultaneous outbreaks of respiratory disease and abortion in domestic pigs that had been occurring since September 2007.[55,117] The clinical presentation resembled a highly virulent strain of porcine reproductive and respiratory syndrome virus, type II (PRRSV) that was circulating in Asia.[117,118] Sera and tissue samples from multiple sites on the island of Luzon were provided. The diagnostic evaluation at FADDL was directed at African swine fever virus, classical swine fever virus, swine vesicular disease virus, and foot and mouth disease virus, as well as PRRSV. The samples tested positive for a pathogenic isolate of a Chinese isolate of PRRSV, and multiple samples were also positive for porcine circovirus type 2 (PCV-2).[55,117] Further investigation for other viral pathogens that may contribute to the atypical presentation of disease was undertaken. A lymph node sample was cultured in Vero cells, an immortalized kidney cell line originating from an NHP, which are non-responsive to endogenous interferon, making them ideal for propagation of numerous viruses. When cell culture evaluation revealed a cytopathic effect, it was determined that a pathogen other than PRRSV was present. It is worth noting that Vero cells are not permissive to the replication of PRRSV, so this finding was highly suggestive of the presence of another virus. Because the potential number of contributing viruses was vast, the sample was queried with a panviral microarray as well as attempts at virus identification via electron microscopy (EM). The results from this broad approach to screening for viral pathogens identified the Ebolavirus genus, primarily highlighting sequences from RESTV with minimal reactivity to other ebolaviruses (Sudan virus and EBOV). Electron microscopy images concurred with the microarray findings by revealing filamentous virus particles.[117]

Because FADDL does not have the capability of operating as a BSL4 facility, samples were immediately transferred to the US Centers for Disease Control in Atlanta, Georgia. To confirm their findings, ebolavirus-specific RT-PCR, antigen-capture enzyme-linked immunosorbent assay, IHC, and further virus isolation in Vero E6 cell culture were performed. This battery of diagnostic assays confirmed the initial findings. RESTV-specific RT-PCR confirmed the presence of positive animals at 3 of the 4 sampled locations in the Philippines, and, interestingly, RESTV was only found in samples that also tested positive for PRRSV. Histopathology and RESTV-specific IHC results were consistent with viral involvement in interstitial pneumonia.[117]

The similarities between the 1998 discovery of RESTV in NHPs in Reston, Virginia, and the 2008 discovery of RESTV in pigs in the Philippines should not be overlooked. Both situations were associated with accidental importation of the pathogen into non-BSL4 facilities in the United States (the first in live animals and the second in animal diagnostic samples), both scenarios originated from within 100 km^2 of each other on the island of Luzon, and both discoveries were associated with animals co-infected with an arterivirus (a simarterivirus in the NHPs and PRRSV in the domestic pigs). Both outbreaks were also associated with asymptomatic infections and subsequent seroconversion in humans determined to have exposure to the infected animals.[117,119] There is a single, well-characterized case of a person that became infected with RESTV after a needle-stick injury sustained during a necropsy of a NHP in 1989. Live virus was isolated from this person's blood at multiple time points.[120] Genomic analysis of the viruses isolated from pigs during the 2008 outbreak showed that the viruses were significantly more divergent from each other than from the 1989 isolate from NHPs, indicating at least 3 independent spillover events occurred from the reservoir host (presumed to be bats) to pigs, leading to sustained pig-to-pig transmission.[117,120] Although RESTV was discovered during the 1989 Reston, Virginia, outbreak in NHPs, the lack of pig isolates phylogenetically distinct from macaque-isolated virus suggests that RESTV isolates from the pigs had been circulating at least since, and possibly before, the 1989 export.[117,120]

Unsurprisingly, this discovery startled the scientific community, and the implications of finding a filovirus in domesticated production animals with potential collateral consequences of an ebolavirus as a possible foodborne pathogen to food security and public health were heavily debated.[121] The seroconversion of people associated with pigs in the Philippines raised concerns of pig-to-human transmission and subsequent infection, even in the absence of clinical disease.[117,119,122,123] Extensive scientific investigations were constrained due to the limitations of available BSL-4 laboratories and the requirements for conducting RG4 research in agriculture animals (BSL-4Ag). At the time, there were only 2 facilities capable of conducting RG4 research with livestock: the AAHL in Geelong, Australia, and the Canadian Food Inspection Agency's NCFAD in Winnipeg, Manitoba, Canada.

Experimental Inoculation of Pigs With RESTV. The isolation of RESTV in naturally infected domestic pigs raised many public health and biosecurity concerns as well as many questions concerning the role the virus played in the observed clinical disease in pigs.[119] The predominate symptoms included fever, coughing, and skin lesions (cyanosis), which could be a consequence of RESTV infection or the result of infection with PRRSV and PCV-2.[43,55,117] To answer these questions and to provide additional information needed for more informed risk assessment and mitigation planning, Marsh et al conducted a series of experiments at the BSL4-Ag facility in Australia.[55] During these experiments, young pigs were inoculated with a Philippine 2008 isolate of RESTV originating from a naturally infected pig by either the oronasal route or subcutaneous injection. The authors confirmed nasopharyngeal shedding of virus through the detection of RNA and by isolation of live virus in the oronasally inoculated group. All pigs, regardless of inoculation method, had virus detected in multiple organs, indicating varying levels of systemic spread. Interestingly, experimental infection did not reproduce the high mortality and severe clinical disease observed during the outbreak in the naturally infected pigs in the Philippines. The researchers raised particular suspicion concerning the potential comorbidity effects of the circulating highly virulent strain of PRRSV, potentially exacerbating the disease caused by RESTV. This study demonstrated that asymptomatic infection with RESTV could occur in pigs. Further, these

asymptomatic animals pose a transmission risk to other pigs and may pose a risk to humans, particularly farm, veterinary, and abattoir-associated workers.[55,119] Marsh et al went on to hypothesize that the differences in virus shedding between study groups could be secondary to subclinical respiratory disease as a result of where the study animal cohorts were sourced. Even though Australia is PRRSV-free and their study animals tested negative for PCV-2, other respiratory pathogens are present in the country's commercial herd.[55]

Recently, Haddock et al performed a second experimental pig inoculation study at the National Institutes of Health's Rocky Mountain Laboratory in Hamilton, Montana, using the same RESTV isolate as Marsh et al and modifying an ABSL-4 facility for livestock use.[43] The overall goal of this study was to investigate potential age-dependent susceptibility to infection and subsequent development of clinical disease. To accomplish this, 3-, 5-, and 7-week-old Yorkshire cross pigs were subjected to oropharyngeal inoculation. This led to the appearance of clinical disease as early as 3 dpi. The clinical presentation progressed from anorexia, depression, hunched posture, and piloerection at 3 dpi in a few animals to severe respiratory distress by 6 dpi in all animals. Respiratory compromise was noted to consist of tachypnea and dyspnea with associated cyanosis. Some animals also had serous nasal discharge and a productive cough, and several animals progressed to early humane endpoints and were subsequently euthanized. Animals surviving the acute phase rapidly recovered to normal clinical status. Systemic infection was evidenced by a detectable viremia in all animals by 7 dpi. Low levels of virus RNA were detected in oral, nasal, and rectal swabs at various time points, with only oral swabs yielding infectious virus. Gross necropsy findings included firm and edematous lungs with enlarged and edematous mediastinal lymph nodes. Further laboratory evaluation revealed high viral loads in the lungs and draining lymph nodes and low viral titers in the liver and spleen. Survivors from the 3-week-old cohort cleared virus by 16 dpi, and the single 5-week-old survivor had detectable virus present in the lungs at 13 dpi.[43]

Even with the drastic differences in clinical outcome between the Marsh et al and Haddock et al studies, it was noted that the tissue tropism and pathology were similar.[43] It was proposed that disease severity was not a factor of age but that the role of pig breed, genetics, or the effect of co-infection with unrelated respiratory or non-respiratory pathogens could not be discerned.[43,55] Haddock et al noted that the inoculation dose they used was 10-fold lower (10^5 compared with 10^6 $TCID_{50}$) and that "over-challenge" of animals, or exposure of study animals to unrealistic doses of inoculum, leading to the initiation of a stronger innate immune response may have attenuated clinical disease in the Marsh et al study.[43] To our knowledge, these are the only scientific studies published involving the experimental inoculation of pigs with RESTV.

Experimental Inoculation of Pigs With EBOV. After the discovery of naturally acquired infection of pigs with RESTV, concerns were raised about other ebolaviruses and the potential consequences of pig involvement in human disease and transmission. As a risk assessment, researchers at the NCFAD, the only facility other than AAHL with BSL4Ag capabilities at the time, conducted experimental inoculations of pigs with an ebolavirus known to cause severe disease in humans. Pigs were inoculated with EBOV (*Zaire ebolavirus*) by the oronasal route combined with ocular inoculation. This virus proved to be far more virulent than RESTV, producing severe respiratory distress in the same age pigs (5–6 weeks old).[51,124] Viral shedding occurred primarily via the respiratory tract, and infectious virus was recovered from both nasal washes and oral swabs from 3 dpi and up to 14 dpi.[124,125] Inoculated pigs shed live virus in nasal wash fluid, which was thought to be at levels sufficient to infect naïve pigs. Because the levels of live virus shed were low, it was proposed that the level of viral shedding suggests within-herd spread in pigs may be self-limiting.[124,126]

Clinical presentation of EBOV infection in pigs ranged from asymptomatic to severe respiratory compromise consisting of increased respiratory rates with labored breathing including an abdominal component. Incubation is suspected to be approximately 3 days, at which point shedding of virus is possible. Development of clinical signs typically occurs a day later. Virus was detected in numerous tissues by 5 and 6 dpi, and gross lesions noted at necropsy were consistent with respiratory involvement, including bronchointerstitial pneumonia.[124,125]

After determining that pigs were susceptible to experimental inoculation and subsequently developed clinical disease, interest turned to determining if infected pigs were capable of successfully transmitting virus by direct contact to naïve pigs that were co-housed in the same room. To accomplish this, a second group of pigs from the same commercial source were inoculated as before then allowed to co-house with uninoculated, naïve pigs. This group of animals was 1 week younger than the first study, and, even though they were able to transmit virus, they developed only mild respiratory disease. Due to the difference in presentation of clinical disease between the 2 studies, a follow-up study used pigs from the same farm and of the same age, but from different sows. The cohort was randomized between 2 groups, with 1 group inoculated at 4 weeks old and the other at 6 weeks old. This study confirmed the previous findings, with the younger pigs developing mild clinical disease and the older pigs progressing to severe respiratory distress.[124]

It is proposed that the immunopathogenesis of EBOV infection in 6-week-old pigs is secondary to over-activation and dysregulation of the proinflammatory response in the lungs. Neutrophils and monocytes/macrophages were involved with the infiltration of the lungs in both age groups of pigs, yet the degree of infiltration was more severe in the older animals. Interestingly, only the macrophages were shown to be infected with virus. The study also noted an upregulation of genes in relation to proinflammatory cytokines, acute-phase proteins, and chemokines in the lungs.[125] The difference in the clinical presentation by age groups was hypothesized to be secondary to the higher levels of IFNγ at the time of inoculation in the younger pigs.[51,124,126]

Experimental Transmission of EBOV From Pigs to NHPs. With the susceptibility of pigs to experimental inoculation with EBOV confirmed, along with experimental transmission between pigs and transmission of RESTV from pigs to farmers in the Philippines, evidence was mounting suggestive of pigs being capable of transmitting EBOV to humans. To investigate this, Weingartl et al, including researchers from the Canadian Food Inspection Agency and Public Health Agency of Canada (PHAC), utilized a NHP model as a human surrogate.[127] Pigs were inoculated oronasally with EBOV and, after inoculation, were transferred to a room housing 4 cynomolgus macaques that were individually housed in an open, yet inaccessible, cage system. The NHP housing was separated from the pig pen by a wire divider to prevent direct contact. Cleaning of the room and handling of the animals was performed in a manner to minimize cross-contamination of

the NHP housing area (eg, low-pressure washing of the floors to prevent splashing).[127,128]

Mild clinical disease was observed in the pigs between 5 and 7 dpi, including increased respiratory rate and increased rectal temperatures (40.2°C–40.5°C). There were no significant lesions noted during necropsy, and histopathology revealed bronchointerstitial pneumonia with a lobular pattern consistent with observations found during previous studies. All 4 macaques were alert and clinically normal until 7 days post exposure (dpe). At 8 dpe, 2 of the NHPs were euthanized after reaching humane endpoints, including petechial hemorrhages of the skin on the sternum as well as along the arms and legs. The remaining animals were clinically normal until 12 dpe and were euthanized at 13 dpe with clinical signs indicative of EBOV infection. During necropsy of the NHPs, gross pathology was apparent in the lungs and the liver. On histopathologic evaluation, an interstitial pneumonia was present consisting of thickened and hypercellular alveolar septa with multifocal areas of alveolar hemorrhage and edema, and extensive EBOV antigen was detected by IHC in both alveolar and septal macrophages. This pattern of lesions and antigen distribution is suggestive of pulmonary infection occurring in the respiratory epithelium as well as viremic spread of the virus.[127] Viremic spread of the virus is consistent with previous NHP studies involving direct inoculation, while the involvement of the respiratory epithelium suggests that this was the route of initial infection and is consistent with previous studies involving aerosol transmission of filoviruses.[127,129] Transmission between infected and naïve macaques housed in similar conditions had never been observed. Together, these findings show EBOV transmission from pigs to cynomolgus macaques without direct contact leading to systemic disease in the NHPs and represented the first study to demonstrate EBOV virus transmission between animals of 2 different species.[127]

Scientific Contributions Toward the Understanding of Filoviruses Made by Experimental Infections of Pigs. Prior to 2013, outbreaks involving the filoviruses were regionally limited, typically occurring in rural and remote locations, with estimates of 2886 human cases and 1982 deaths since 1967.[116] The most extensive outbreak occurred in the Gulu district of Uganda in 2000–2002, ultimately involving 425 human cases and 224 deaths due to Sudan virus.[130,131] Regarding global public health, it was typically assumed that EBOV, as well as the other filoviruses, was an exotic pathogen of negligible consequences for those outside of Middle Africa. This perspective quickly changed with the 2013–2016 Western Africa outbreak. After emergence in Guinea, cases spread from remote areas to populous cities in Africa and, eventually, sourced imported cases throughout the world, leading to 28 652 human cases and 11 325 deaths. Health-care systems and economies were virtually destroyed in some of the affected countries.[116,132–134]

Most outbreaks of filoviruses can be attributed to a single spillover event originating from an unknown reservoir that leads to human-to-human transmission. Typically, this sole event leads to infection of an index case and then human-to-human spread via direct or close contact with infected persons. Contact with body fluids or tissues (eg, care of the sick, funeral and burial practices) or fomite transmission are the most common means of transmission. Though a lot is known about transmission after infection of the index case, how the index case gets infected from the reservoir remains a mystery.[116,132] The findings from experimental studies conducted in BSL4-Ag facilities with pigs encouraged domestic animal surveillance studies in areas with a history of EBOV endemicity (predominately Middle and Western Africa as well as the Philippines) and areas that are part of, or adjacent to, bat flyways (China).[135–138] A report from China confirms the existence of RESTV antibodies in pigs on production farms in the area of Shanghai, extending the potential endemic region of the virus in Asia.[138]

RESTV has not caused disease in humans, and it is, at this time, not considered a significant threat to human public health. Due to the lack of understanding concerning the mechanisms of attenuation and reasons for the lack of pathogenesis in humans, RESTV is still classified as a RG4 agent.[119] The finding of natural susceptibility and pig-to-pig transmission raises the concern that sustained passage in domesticated pigs could result in virus mutation resulting in the emergence of pathogenic strains capable of causing disease in humans. Therefore, advancing scientific understanding of RESTV and furthering efforts in the identification and control of outbreaks is fundamental to human public health, animal health, and food security.[43,126]

At this time, there are only reports of experimental infections of pigs with 2 of the known ebolaviruses, EBOV and RESTV.[43,55,124] From these studies, it has been shown that infection in domestic pigs is systemic with major involvement of the respiratory tract, including direct pathology of the lungs, allowing transmission between pigs and, at least for EBOV, transmission from pigs to NHPs.[127] If NHPs are seen as surrogates for humans, these findings suggest that pigs may be capable of transmission of virus to humans and this risk could be heightened in a farm setting. This conclusion is supported by the RESTV seropositivity of pig farmers in the Philippines that reported no involvement with either slaughter or contact with contaminated pig tissues.[43,117,119,127,139] Experimental inoculations of pigs has also raised the possibility that pigs, either wild or domestic, may play a role in EBOV's natural life cycle in sub-Saharan Africa.[43,127]

Even after over 50 years of research, the filoviruses are still an enigma in many ways. There are many ecological uncertainties that remain to be clarified for ebolaviruses, including determining the identity of the natural reservoir host, presumed to be bats, and determining the role that other animals may play in the dynamics of spillover and subsequent human-to-human transmission. Experimental studies with domestic pigs including both EBOV and RESTV have offered insights into filoviral pathogenesis. Understanding the role that pigs, a domesticated animal involved in intensive production and a major source of dietary protein throughout the world, may play is paramount to risk assessments concerning public health and food security. Advancing the understanding of the dynamics of the animal–human interface may help prevent or limit the impact of future emerging diseases on the human population.

CONCLUSIONS AND THE FUTURE OF LIVESTOCK RESEARCH IN MAXIMUM CONTAINMENT

Biocontainment laboratories are a unique component of the bioeconomy of the United States and play a critical role in the national infrastructure, supporting research on a select set of especially dangerous pathogens as well as novel, emerging diseases.[40] Historically, the maximum containment research community is composed of a small, close-knit group of highly specialized researchers.[42] As we move forward, we need to continue to be transparent while enhancing the general public perspective and understanding of our work and the need for continued progress, because lack of public confidence can become a

significant barrier.[140] Maximum containment research is notoriously secluded, most likely as a consequence of many factors, including expanding select agent regulations and the propensity of the research to be considered to have dual-use purposes.[40] International engagement between government organizations is currently improving and expanding through multisectoral groups. The Biosafety Level 4 Zoonotic Laboratory Network is one of these groups that functions to enhance knowledge, competency, and capacity building to meet the current and future needs of the maximum containment community.[141] We also need to make our goals and achievements known to our peers within the animal research community while improving our outreach and collaboration with these colleagues to benefit from their knowledge and experience.

Well-designed research provides a sound basis for risk assessments and policy decisions on how to deploy limited resources in the time of need.[11] Continued use of animal models, and continued expansion of these models to include available livestock of various species, is essential to deciphering the ecology of emerging and re-emerging infectious diseases as well as for emergency response and mitigation preparedness.

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