# Biopreserving Pathogens: Promise & Peril

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**Abstract:** The development of technologies for the biopreservation of infectious organisms requires careful analysis of benefits and risks. This article reviews the regulatory landscape and oversight responsibilities in the United States in respect to pathogen biopreservation. Focused on two globally significant pathogens, *Cryptosporidium* and *Plasmodium*, the article explores advantages and potential risks of biopreservation concerning biosafety, biosecurity and biocontainment.

## Introduction

Biopreservation plays an important role in pathogen research by enabling biobanking of wildtype and genetically modified strains and isolates of infectious organisms associated with human health. Pathogen biopreservation further allows storage and sharing of quality-controlled specimens to facilitate the standardization of procedures, which are pivotal in experimental research and clinical trials for the development of life-saving vaccines and drugs. Similarly, these benefits extend to animal health, agriculture, and environment, where preservation may aid in disease management, crop protection, and wildlife conservation. However, it is essential to consider potential risks associated with pathogen preservation, particularly concerning biosafety, biosecurity, and biocontainment. This is particularly true for infectious organisms, which carry an inherent risk of infection, transmission, and spread. Here, safety, ethical, legal, and societal issues must be considered given the potential impact on human and animal health as well as environmental health and safety. The development and

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deployment of technologies for the biopreservation of infectious agents must therefore be accompanied by strategies to understand and mitigate these potential risks. A rigorous oversight mechanism can facilitate responsible and ethical management, to help ensure that benefits outweigh the risks.

There is a robust body of literature concerning the regulatory and institutional oversight of research posing biological risks. However, emerging technologies that enable the long-term storage of pathogenic organisms under cryogenic conditions warrant further consideration. Here, we analyze the benefits and risks, applicable policy framework, and oversight responsibilities that should be considered when conducting research and developing technologies for the biopreservation of infectious organisms. Although we recognize that pathogen biopreservation raises international issues, here we focus on the US policy framework. Pathogens crossing international borders activate complex mechanisms of export control and international health regulations and therefore warrant separate attention.

We specifically consider the preservation of two apicomplexan parasitic organisms of global health importance that are being studied at the NSF Engineering Research Center (ERC) for Advanced Technologies for the Preservation of Biological Systems (ATP-Bio), namely *Cryptosporidium* and *Plasmodium*. In the last decade these two pathogens were responsible for an estimated 250 million human infections annually across the globe — a malarial disease caused by *Plasmodium*[1] and a diarrheal disease caused by *Cryptosporidium*.[2] Both diseases constitute a potential threat to the US public health.[3] The overwhelming disease burden underscores the need for research to develop new therapeutics and vaccines against these diseases. Biopreservation technologies developed for *Plasmodium* and *Cryptosporidium* organisms offer significant opportunities to advance research on each disease. However, placing infectious agents in suspended animation, storing them over time and transporting them over space, requires risk management and warrants establishment of anticipatory governance. Identification of oversight gaps associated with biopreservation is crucial to mitigate the potential risks and ensure they are outweighed by the benefits. This is especially critical for pathogens posing risks to biosecurity, such as the zoonotic species of the *Cryptosporidium* parasite. Here, we leverage the different biosecurity profiles of *Plasmodium* and *Cryptosporidium* to analyze the benefits and risks related to pathogen cryopreservation and recommend oversight strategies. *Plasmodium* offers a case study of a pathogen preserved for decades through traditional methods, underscoring the advantages for research and medicine. In contrast, *Cryptosporidium* research has been stymied by lack of preservation methods, and cryopreservation employing novel technologies represents an emerging and swiftly evolving field, where oversight is warranted.

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## I. *Plasmodium and Cryptosporidium:* Biology and Biopreservation

*Plasmodium* and *Cryptosporidium* are apicomplexan parasites characterized by distinct tissue tropism and host specificities, determining their unique transmissibility and infectivity.[4] Both parasites have adapted to a range of vertebrate hosts, diverging into host-specific species. While the monoxenous life cycle of *Cryptosporidium* requires only one host, the *Plasmodium* life cycle is heteroxenous and employs a mosquito vector for transmission between vertebrate hosts.

The life cycle of both parasite genera occurs in the intracellular niche; *Cryptosporidium* develops in the



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epithelium of the gastrointestinal tract, while *Plasmodium* develops in hepatocytes and red blood cells of the vertebrate host and a midgut epithelium of the invertebrate vector. Within the vertebrate host, only a few life stages appear briefly in the extracellular space between events of cell invasion, specifically in the gut lumen for *Cryptosporidium* or blood plasma for *Plasmodium*. This distinct tissue tropism dictates unique pathology and symptomology in the vertebrate host, a diarrheal disease in the case of cryptosporidiosis and a systemic multi-organ disease in case of malaria. Related to the distinct tissue tropism is the path by which the pathogen evacuates from the host, which defines the risk of disease transmission and spread within the population. These differences between life cycles of the two parasites contribute to their respective biosecurity profiles in the context of biopreservation.

Infection with *Cryptosporidium* leads to an acute diarrheal disease in animals and in humans. Most human cryptosporidiosis is caused by the anthroponotic *C. hominis* and zoonotic *C. parvum* circulating in cattle. Both species transmit between hosts by the fecal-oral route, including waterborne, foodborne, person-to-person, and animal-to-person modes of transmission. After completion of a life cycle in the intestine, *Cryptosporidium* evacuates its host with feces in the form of a free-living oocyst. Encapsulated in a thick protective wall, oocysts remain dormant in the environment for several weeks awaiting consumption by the next host. Millions of oocysts can evacuate during a single fecal discharge[5] leading to a substantial contamination of the environment, soil, and groundwater. *Cryptosporidium* is extremely infectious; consumption of as few as 10 oocysts can lead to infection.[6] As a result of their resistance to chemical disinfectants and their small size allowing for filtration bypass, oocysts persist in the environment, giving rise to frequent outbreaks associated with waterborne transmission via ingestion of recreational[7] or consumable water.[8] Foodborne outbreaks via consumption of fecally contaminated food have also been reported.[9] Additionally, infection among farmed cattle exponentially amplifies the environmental parasite burden and exacerbates the risk of large zoonotic outbreaks.[10]

The largest cryptosporidiosis outbreak in humans occurred in the United States and was caused by *C. parvum*. The outbreak was associated with contamination of water by agricultural runoff, leading to an estimated 200,000 infections and a breakdown of the state healthcare system.[11] The growing number of US cryptosporidiosis outbreaks in the last decade is concerning as there are limited therapeutics and no vaccines available.[12] Given the potential of oocyst dissemination via water, high morbidity rates, and the need for specific diagnostic capacity, the Centers for Disease Control and Prevention (CDC) designate *C. parvum* as a bioterrorism agent Category B.[13]

Although infectious, the *Plasmodium* parasite poses a less immediate biosecurity risk as the life cycle does not include a free-living infectious form and instead relies on continuous circulation between various vertebrate hosts and a mosquito vector.[14] Five malaria parasite species are responsible for human disease, putting almost half the world's population at risk.[15] Transmission to humans occurs when an infected *Anopheles* mosquito injects *Plasmodium* sporozoites into the bloodstream while taking a blood meal.

Chronicity of infection in the host and the abundance of mosquitoes perpetuate the disease burden in several regions.[16] Although rare, human-to-human malaria transmission has also occurred via blood transfusions, sharing contaminated needles, solid organ transplantation, and transplacental transmission.[17] Approaches to reduce the malaria burden include vector control, implementation of antimalarial drugs, and recently vaccine prevention.[18] Due to the parenteral access required for disease transmission and availability of effective treatments and prevention, the biosecurity risk associated with *Plasmodium* research in a laboratory setting is greatly reduced.

Cryopreservation methods have been established for both *Plasmodium* and *Cryptosporidium* parasites. In case of *P. falciparum*, methods of cryogenic preservation of asexual intracellular blood stages have been widely available for decades and are useful for the preservation of both in vitro cultured parasites and clinical isolates obtained from patient samples.[19] These methods rely on principles of slow cooling in the presence of cryoprotective agents (CPAs). In an effort to improve access to quality-controlled *Plasmodium* parasites, the National Institute of Allergy and Infectious Disease (NIAID) at the National Institutes of Health (NIH) convened The Malaria Research and Reference Reagent Resource Center (MR4), which is currently responsible for cryobanking and sharing the asexual blood stages of multiple strains of *Plasmodium* species. Methods for preservation of sexual blood stages and extracellular sporozoites by slow cooling have been previously reported,[20] but are not yet widely used in laboratory practice.

In contrast, traditional slow cooling methods have not been successfully applied for the cryopreservation of *Cryptosporidium* parasites, despite extensive efforts. This failure is likely due to the impermeability of the oocyst wall to CPAs to impart protection to the oocyst and the sensitivity of the parasite to ice crys-



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tallization. Only in recent years has cryopreservation of infectious *Cryptosporidium* been accomplished by application of newer cryopreservation technologies, namely by vitrification.[21] Vitrification is an ice-free approach to cryopreservation where relatively high concentrations of CPAs are combined with rapid cooling rates to achieve formation of amorphous solidification in a glassy state as opposed to crystalline formation of ice. Vitrification of various species of infectious *Cryptosporidium* parasites by ultra-rapid cooling has been achieved using highly thermally conductive silica microcapillaries.[22] Due to the small volume restrictions imposed by microcapillaries, additional technologies utilizing high aspect ratio specimen containers have been developed to successfully cryopreserve larger volumes (~100 μL) of *C. hominis* and *C. parvum*.[23] However, cryopreservation of other parasitic stages, namely extracellular sporozoites and intracellular stages, has not yet been achieved.

Advanced technologies for biopreservation have the potential to be a game changer in basic and translational research on pathogens. However, biopreservation of infectious pathogens requires balancing benefits and risks, as well as appropriate governance. Below we discuss cryopreservation of *Plasmodium* and *Cryptosporidium* as model pathogens of differing risk to biosecurity, to offer a recommended oversight framework.

## II. Benefits of Pathogen Cryopreservation

Cryopreservation is broadly utilized in scientific and clinical research for the purpose of preserving biological materials ranging from cells to tissues and even small organisms. Cryopreservation relieves logistical challenges by allowing researchers to bank biospecimens for dissemination or for later use. In infectious disease research, access to reference organisms is fundamental to studying pathogen biology and host-pathogen interactions, discovering therapeutics and vaccines, evaluating those interventions in preclinical and clinical trials, and providing standards for diagnostics and outbreak investigations.

Pathogen species exist in nature in a multitude of strains, each characterized by distinct genetic markers, antigenic properties, or other unique traits that distinguish them from one another. To standardize a pathogen strain for research purposes, a wild-type isolate is first obtained from an infected host or the environment. This is followed by laboratory adaptation to in vitro and in vivo systems designed to replicate the pathogen and mirror its life cycle in a host. Additionally, standardized pathogens are genetically altered to gain or eliminate function, giving rise to transgenic and mutant varieties. This process generates a considerable inventory of laboratory-adapted pathogen species and strains, each of them unique and valuable to research. Biobanking these pathogens is vital to the continuity of research and collective progress, as it safeguards unique organisms and enables access and sharing with the broader scientific community.

A large number of *Plasmodium* strains are in existence in laboratories around the world. The Malaria Research and Reference Reagent Resource Center (MR4) alone biobanks 250 unique *Plasmodium* strains,[24] facilitating centralized access to quality-controlled and authenticated materials that are critical within the malaria research community and beyond. *P. falciparum* blood stages can be maintained in in vitro cultures using red blood cells. Further, the entire parasite lifecycle of *P. falciparum* and other species can be generated from infection of a species-specific vertebrate host and a mosquito vector. While *P. falciparum* blood stages can be effectively cultured in vitro, prolonged cultivation may curb progression to sexual differentiation, making use of an animal model and the mosquito vector indispensable for maintenance of parasite strains.[25] However, the vertebrate animal models for cultivation of human species of malaria are expensive and not readily accessible (i.e., non-human primate and humanized mouse models), making maintenance costly and restricted to specialized laboratories.[26] Further, to mimic a natural infection of the host via a mosquito bite, a live infected mosquito vector is necessary to isolate fresh sporozoite stages. The mosquito vector is typically procured by transport from specialized facilities. Transit of *Plasmodium*-infected mosquitos is vulnerable to breach of safety standards and therefore carries a risk of accidental release of the pathogen and non-native species of mosquitoes to the environment.

In contrast to *Plasmodium*, only a limited number of laboratory strains of *Cryptosporidium* are maintained worldwide, including a single human isolate of *C. hominis* and a few bovine isolates of *C. parvum*. However, the number of *C. parvum* isolates has grown with the emergence of methods for genetic manipulation.[27] Maintenance of *Cryptosporidium* isolates entails serial propagation in a single susceptible animal host, specifically mice and neonatal calves for *C. parvum*[28] and gnotobiotic piglets for *C. hominis*.[29] Infected animals develop gastrointestinal illness and release oocysts which can be purified from feces. Due to the short shelf life of oocysts, animal passage is scheduled 4–6 times per year, an expensive and time-consuming process. Continuous generation of *C. hominis* in gnotobiotic piglets is particularly labor-



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intensive as it involves surgical derivation via Cesarean section and housing of piglets in sterile isolators, and therefore requires access to specialized facilities and trained veterinary personnel.

The rigors of maintaining pathogenic specimens in a laboratory creates a major barrier to research due to technical difficulties and inherent risks. In the case of *Plasmodium* and *Cryptosporidium*, the ability to cryogenically bank large quantities of strains can significantly reduce the labor and cost associated with continuous maintenance of reference laboratory organisms. Another benefit to cryopreservation is a reduction in the number of animals used for propagation, thus improving animal welfare under the ethical framework of the Three Rs principle, which promotes replacement, reduction and refinement of animal

Although discovered two decades apart, research on *Cryptosporidium* lags in comparison to that on *Plasmodium*, and this delay may be partially attributed to many technical limitations, including lacking access to cryobanking. The first method of *Cryptosporidium* cryopreservation was achieved only recently, while methods of *Plasmodium* cryopreservation have been routinely in use for five decades. This lag coincides with the delay in scientific discovery as the search for effective drugs and vaccines against cryptosporidiosis continues.

participation in experimental studies.[30] Additionally, cryopreservation can maintain genetic characteristics that may otherwise be altered as a result of prolonged cultivation or propagation. Streamlined cryopreservation also enables creation of pathogen biorepositories to serve as a centralized source for dissemination of specimens. This enables wider access to biological specimens, improves sharing between laboratories and importantly, extends access to researchers lacking capacity for routine maintenance of host species. Because cryopreservation enables on-demand availability as opposed to continuous culture, this technology also increases the number of clinical isolates and strains that can be studied, thus accelerating potential research. Cryopreservation is also a potential strategy to reduce the risk of accidental release of infectious specimens during transport, as typically a precise protocol for recovery from the frozen state must be executed to preserve infectivity. In the case of *Plasmodium* sporozoites, transport of live malaria-infected mosquitoes could be entirely avoided with streamlined methods of sporozoite preservation, eliminating the risk of pathogen and vector release to the environment. Further, biobanking of unique pathogen isolates and strains protects from catastrophic specimen loss due to unforeseen events such as pandemics, natural disasters, and associated disruptions to the supply chain.

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In drug and vaccine development, pathogen cryopreservation is an important enabling technology with applications in research and clinical studies. For example, performing controlled human infection models (CHIM) or early clinical trials require utilization of a quality-controlled pathogen source across study subjects to ensure uniformity of the infectious dose. However, each passage of a pathogen in an animal or cell culture may alter pathogen characteristics such as infectivity and virulence and give rise to between-batch variation. Therefore, each propagated batch demands optimization, standardization, and in vitro validation prior to use in clinical trials. The ability to cryobank multiple inocula from a single standardized source allows researchers to thaw an individual infectious dose on an as-needed basis, which can eliminate variability and improve the uniformity of infection under trial conditions.[34] In the case of infectious organisms that predominantly affect persons living in low-and middle-income countries or that exhibit seasonal patterns of infection, cryopreservation enables routine access to research specimens globally. The development of biopreservation technologies for infec-



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tious organisms can thus advance research to develop therapeutics and vaccines, ultimately yielding health, societal, and economic benefits through the relief of disease burden and improvement of social equity.[35]

Lastly, cryopreservation is essential for the long-term preservation of eradicated pathogen species (e.g., smallpox and rinderpest viruses or eradicated European *P. falciparum* strain[36]). This secures the opportunities for continuation of scientific research, diagnostics, drug and vaccine development in the event of resurgence of these diseases but without the risks related to the maintenance of live cultures. However, special safety considerations should be observed during the cryopreservation of eradicated pathogens to prevent accidental release and potential reemergence.

## III. Risks of Pathogen Cryopreservation

While pathogen cryopreservation promises significant benefits, it may also carry risks over time and space. Although handling of both *Plasmodium* and *Cryptosporidium* pathogens requires compliance with biosafety level 2 (BSL-2) standards, these seemingly similar pathogens carry different degrees of biological risk. Due to the potential for waterborne transmission, *Cryptosporidium* poses a threat to public health, hence its classification by CDC as a Category B bioterrorism agent.[37] Its release in the environment, whether accidental or intentional, can lead to contamination of drinking or recreational water and result in large outbreaks, potentially affecting thousands of people in a short period of time.[38] In contrast, transmission of *Plasmodium* relies on blood-to-blood contact, whether by injection or mosquito bite, which — in tandem with existing preventative and therapeutic solutions — considerably reduces the risks associated with release of biospecimens to the population in relation to cryopreservation. Consequently, in this section we will focus solely on potential perils associated with *Cryptosporidium* cryopreservation as an example of a pathogen with high biological risk, defined here as having the potential to cause human harm.[39]

Biological risk refers to the potential threat or danger posed by biological agents that have the capacity to cause harm to human health, the environment, or other living organisms. Biological agents include microorganisms, toxins, genetically modified organisms, and prions. Risk is inherent to the laboratory handling of biological agents and is therefore rigidly managed. Strategies for mitigating biological risks include implementation of biosafety, biosecurity, and biocontainment procedures to prevent accidental exposures, intentional misuse, or accidental release of biological agents. Although biosafety, biosecurity, and biocontainment are related concepts, they refer to different aspects of biological material handling.[40] Biosafety refers to the set of practices designed to protect laboratory workers from accidental exposure to biological agents and their further release to the environment. Biosecurity involves measures preventing unauthorized access, theft, loss, or intentional release of hazardous biological materials. Its primary focus is therefore on safeguarding biological materials and associated information from intentional misuse, whether by individuals, groups, or nations. Lastly, biocontainment refers to the physical and procedural barriers implemented to confine biological agents to a controlled space and prevent their escape into the surrounding environment. Together, these measures form a comprehensive framework that ensures responsible and safe practices in the field of biological research and protects the public and the environment from introduction of biological agents.

Standard laboratory handling of *Cryptosporidium* carries risks relating to biosafety, biosecurity, and biocontainment. Technologies to cryopreserve *Cryptosporidium* may increase the risk of inadvertent pathogen escape, whether accidental or intentional, under certain conditions. Thus, utilization of advanced technologies for pathogen preservation should proactively identify these elevated risks. Further, implementation of these advanced technologies in pathogen cryopreservation may inherently elevate biosafety risk. For example, the technologies developed for vitrification of *Cryptosporidium* oocysts involve use of devices and procedures prone to pressurization and aerosolization, and therefore introduce a risk of unintentional release through an exposed laboratory worker.[41] It is therefore critical that both cooling and thawing, events separated in time and likely in space, are performed by skilled and trained personnel in an appropriate BSL environment.

Pathogen cryopreservation carries the potential to undermine biosecurity measures. This could present a potential for misuse, deliberate release, or theft, raising concerns about illicit experimentation, biowarfare, or other malicious activities. Intentional misuse is especially relevant to *C. parvum* due to its recognition as a bioterrorism agent. A major concern is that biorepositories of pathogens and associated information may be vulnerable to unauthorized access, therefore implementation of robust security measures is essential to mitigate such risk. The security protocols established for live pathogens should be extended to encompass cryopreserved specimens, securing both the physical specimens and the digital information about those specimens. The long-term nature of cryo-



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genic storage introduces challenges in consistently maintaining and enforcing strict biosecurity protocols over extended periods of time. This could be due to employee turnover or poor recordkeeping, which could allow sensitive materials to be accidentally relocated to unsecured facilities.

Pathogen cryopreservation introduces potential biocontainment challenges as well, primarily by altering the dynamics of pathogen storage. Cryogenic storage of pathogens could lead to a risk of inadvertent release or exposure during thawing. Release of *Cryptosporidium* oocysts, even in limited quantities, poses a significant threat to individuals who come into contact with the contaminated environment. This necessitates stringent biocontainment measures during the handling and storage of such pathogens. The transport of cryopreserved materials further amplifies this vulnerability. A breach in biocontainment during static cryostorage or transport could result from infrastructure failures, equipment malfunction, unexpected environmental conditions, human error, or inadequate adherence to protocols. Maintaining biocontainment becomes especially challenging when utilizing emerging preservation technologies which sometimes rely on specimen containers that are either open to the environment or only partially contained, such as microcapillary,[42] droplet[43] or mesh[44] used for vitrification. To address these concerns, comprehensive risk assessments, rigorous personnel training programs, and careful adherence to established biosafety protocols are essential.

The primary objective of cryopreserving pathogens is to maintain their viability for research or storage. However, the inherent stress imposed by freezing and thawing can induce various changes in the organisms i.e., genetic mutations, epigenetic alterations, and thus adaptation to selective pressures, which has been observed in other microorganisms.[45] Though not previously reported, these changes could potentially promote the survival of variants tolerant to cryopreservation stresses and result in novel phenotypes exhibiting modified functions in terms of virulence or drug sensitivity. A comprehensive understanding of the molecular mechanisms underlying these alterations should be a future direction for the field to study. This information can help inform the establishment of biosafety, biosecurity, and biocontainment measures to reduce the impact of these variants and prevent their release into the environment.

## IV. Existing Oversight Framework in Pathogen Research

The US policy framework on pathogen handling, whether live or biopreserved, involves a combination of laws, regulations, guidelines, and oversight mechanisms at the federal, state, and institutional levels. The primary goal is to ensure the safe and responsible handling of pathogens to protect public health and prevent accidental releases and intentional misuse.

### *Federal and State Oversight*

Various federal agencies and departments play a role in governing the handling and research of infectious organisms based on their areas of expertise and mandates. If the management of pathogens is related to public health, it is within the regulatory purview of the Department of Health and Human Services (DHHS). The Public Health Service Act (PHSA) of 1944 provides legal authority for DHHS to regulate and oversee all public health matters, including those related to infectious diseases.[46] The health agencies granted authority by PHSA over infectious disease control operate under DHHS, specifically the CDC,[47] the NIH,[48] and the Food and Drug Administration (FDA).[49] As the primary authority in the field of public health, the CDC offers recommendations regarding the management, confinement, and transportation of infectious agents. CDC issues directives pertaining to biosafety and biosecurity in research laboratories, explicitly addressing biosafety levels. As the primary agency for biomedical research, the NIH actively engages in the support of infectious disease research. It plays a role in establishing guidelines and standards for the conduct of research and specific standards for biosafety and biosecurity in laboratories. When the handling of pathogens pertains to food and medicines, it falls under the jurisdiction of the FDA. This includes development and enforcement of guidance to researchers and manufacturers to facilitate the development of safe and effective pharmaceutical products and unadulterated food. In matters related to the research and utilization of pathogens in agriculture, oversight falls to the United States Department of Agriculture (USDA). Under the Animal Health Protection Act (AHPA) of 2002, the USDA is responsible for regulating activities that involve pathogens that may affect crops, livestock, and other components of the agricultural ecosystem.[50] Through the Animal and Plant Health Inspection Service (APHIS), the USDA is involved in development of biocontainment and biosafety measures for research facilities working with pathogens that could have implications for agriculture.



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Research involving pathogens is addressed under the Dual Use Research of Concern (DURC) framework initiated by the NIH.[51] The DURC framework recognizes the potential for both beneficial and harmful pathogen research that could impact public health, agriculture, the environment, or national security. The framework specifies guidelines and oversight mechanisms to address the responsible conduct of research with dual-use potential. Researchers receiving federal funding are required to adhere to these policies, which include risk assessment and the development of risk mitigation plans. Per this requirement, on the institutional level, the DURC framework delegates the oversight of relevant research to the Institutional Biosafety Committee (IBC).

With respect to national security and defense, the Department of Homeland Security (DHS) and the Department of Defense (DoD) regulate pathogen research. The Homeland Security Act of 2002 delegates protection against and response to biological threats to the DHS.[52] Specifically, the Countering Weapons of Mass Destruction (CWMD) Office within the DHS oversees research involving infectious agents.[53] The role of DoD in the regulation of biological research is to deter the utilization of pathogens for malicious purposes and is outlined by the Biological Weapons Convention treaty of 1972.[54] Oversight by the DHS and the DoD ensures that research activities involving these agents adhere to strict safety and security standards. Further, the USDA and CDC jointly oversee the Federal Select Agent Program (FSAP) under the Public Health Security and Bioterrorism Preparedness and Response Act of 2002.[55] FSAP is a framework for supervising and regulating the possession, use, and transfer of select agents and toxins that could potentially pose significant threats to public health and safety.[56] The agencies collaborate to create the list of select agents and toxins, receiving input from DHS and DoD. Specific to long-term storage of select agents, CDC ensures that storage location and personnel access are compliant with FSAP.[57] Bioterrorism agents encompass a wider range of biological agents, whether or not they are officially designated as select agents. Bioterrorism agents are classified based on the intent to cause harm, panic, or disruption, often in a deliberate act of terrorism. In the United States, key agencies involved in the categorization and assessment of bioterrorism agents include the CDC, DHS, Federal Bureau of Investigation (FBI), DoD, and state and local public health departments and agencies.

Promulgation of biosafety standards to promote the safety and health of workers engaged in infectious disease research is the responsibility of the Occupational Safety and Health Administration (OSHA), a federal agency operating within the Department of Labor under the OSH Act of 1970.[58] OSHA provides guidelines for personal protective equipment, training, and facility design.

When infectious agents are transported, the Department of Transportation (DOT) ensures regulatory compliance. The DOT regulates domestic shipping and transportation of hazardous materials as outlined in the Hazardous Materials Regulations (HMR).[59] The regulations aim to ensure biocontainment and biosecurity. The DOT works in conjunction with the CDC, USDA, and DHS regarding the shipping of pathogens depending on their risk profile.

In managing biocontainment and biosecurity, the Environmental Protection Agency (EPA) takes on the crucial responsibility of regulating the proper disposal of infectious waste. While the disposal of general medical waste is primarily regulated at the state level, the federal Resource Conservation and Recovery Act (RCRA) provides a framework for managing hazardous waste, including some categories of infectious waste.[60] RCRA grants EPA the authority to regulate the generation, transportation, treatment, storage, and disposal of hazardous waste.

Conducting pathogen research also requires compliance with state-level regulatory frameworks. The specific regulations may differ across states, but typically involve collaboration with the state health departments, state environmental agencies, and state agencies related to planning or land management.

*Institutional/Industrial Responsibility*

Pathogen research is subject to additional oversight mechanisms at the institutional level to ensure the safety of researchers, the community, and the environment. Each institution receiving federal funding and conducting research with recombinant DNA or biohazardous materials, including pathogens, is responsible for establishing and maintaining an IBC. IBCs must operate in keeping with the NIH Guidelines and are overseen by the NIH Office of Science Policy (OSP).[61] IBCs are responsible for reviewing and approving research protocols to ensure compliance with the NIH Guidelines and ethical considerations. If the research on pathogens involves human or animal participants/subjects, additional oversight by the Institutional Review Board (IRB) or the Institutional Animal Care and Use Committee (IACUC) is required, respectively. Although distinct, the IBC, IRB, and IACUC serve complementary roles in the oversight of pathogen research.[62] The IACUC is mandated by the Animal Welfare Act (AWA) and ensures



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the ethical and humane treatment of animals used in research and teaching.[63] The IRB is mandated by the Federal Policy for the Protection of Human Subjects (part of which is also known as the Common Rule) and analogous FDA regulations to protect the rights, safety, and well-being of human subjects participating in research.[64] Both the IRB and IACUC consider the potential risks associated with the use of pathogens when reviewing and approving research protocols.

Industry is also subjected to compliance with regulations and standards and is therefore required to develop internal oversight mechanisms. For instance, the guidelines for handling of pathogens in manufacturing settings are outlined by Good Laboratory Practices (GLP) and Good Manufacturing Practices (GMP).[65] Specific to air transport, the International Air Transport Association (IATA) develops industry guidelines for transport of infectious materials as outlined in the IATA Dangerous Goods Regulations (DGR).[66]

Overall, the combination of federal, state, and institutional oversight mechanisms helps create a system that regulates and monitors pathogen handling activities in the United States, including those related to pathogen biopreservation. The goal is to strike a balance between promoting scientific research and safeguarding public health and national security.

## V. Recommendations

Biopreserving pathogens extends their reach and persistence in time and space, emphasizing the need for protective measures. While the US policy framework concerning pathogen handling comprehensively addresses various aspects of biopreservation, research involving the development and application of protocols to preserve, transport, and store pathogens require attention to several issues. These include considering the perils of sharing protocols for the biopreservation of dangerous pathogens, ensuring proper training of personnel handling pathogens, ensuring the security and compliance of facilities housing pathogens, verifying safe transport, developing and monitoring standard operating procedures (SOPs) over time, and building systems to detect and promptly address breaches.

Publishing protocols for cryopreservation of pathogens raises the classic dual-use problem of enabling beneficial research but potentially also empowering laboratories with inadequate biosafety measures as well as malicious actors to cause harm inadvertently or deliberately. A version of this problem arose nearly a decade ago in debate over publishing gain-of-function (GOF) protocols involving pathogens with pandemic potential (P3) in the open scientific literature.[67] Although there are clear differences between protocols that may increase the danger posed by P3 versus protocols to permit pathogen biopreservation, transport, and storage, both may be a source of risk if laboratories failing to take appropriate precautions or malevolent actors intentionally use the pathogens to cause harm. In the GOF context, although the US National Science Advisory Board for Biosecurity (NSABB) initially recommended the publication of these studies in a redacted form, with key findings included but detailed descriptions of materials and methods omitted, they later approved the release of full information.[68] This decision was influenced by a risk/benefit analysis and a recognition of the challenges in reconciling redacted or classified publication with the norms and practicalities of open science. Cryopreservation of pathogens promises the substantial research benefits outlined above, such as aiding development of treatments and vaccines, thus outweighing potential risks associated with open literature access.

Although the dangers of careless or deliberate misuse of *Cryptosporidium* (the more dangerous of the two pathogens we review) fall short of P3, its cryopreservation requires safeguards, and the level of prophylaxis required could be greater for more dangerous pathogens. Those safeguards should include, but are not limited to:

- **Training**: Appropriate training should be in place for all personnel working within a space that houses cryopreserved infectious specimens. This includes institution-based training but must also involve direct training by the laboratory director in pathogen-specific SOPs for the storage of cryopreserved infectious materials. This includes training regarding PPE, spill procedures, risk assessment, safety reporting procedures, recordkeeping and appropriate handling techniques when accessing the storage tank or thawing specimens. Careful adherence to established protocols is essential for maintenance of biosafety and biocontainment.
- **Biosafety**: Biorepositories with infectious materials should be maintained in an appropriate BSL facility accessible only by authorized personnel (i.e., storage of cryopreserved stocks of *Cryptosporidium* should remain in BSL-2). For pathogens stored in the liquid phase of nitrogen, cross-contamination within a storage unit should be prevented and steps should be taken if a vial containing the pathogen ruptures.[69] While pathogens liberated from cryostorage without an



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appropriate thawing technique may not survive, any materials exiting a tank should be considered potentially infectious, as only a few surviving pathogens could in theory cause infection.

- **Technical handling:** Use of advanced technologies for cryopreservation may increase risks to staff. For example, vitrification of *Cryptosporidium* in glass microcapillaries and high aspect ratio cassettes creates additional risks related to pressurization and exposure to sharps. Detailed protocols should be published for these methods, with stress on the importance of proper use of PPE, engineering controls and practices (e.g., expelling contents directly into liquid to prevent aerosolization or use of blunt-tip and safety needles to reduce opportunity for sharp injury).[70] The design of specific cryopreservation technologies should also prioritize safety of the end user. For example, the microcapillary for vitrification of *Cryptosporidium* utilizes a fused silica material, which was selected due to its high resistance to cracking under thermal shock.[71] The risk of sharp injury was further reduced by utilization of microcapillaries coated with polyimide, which increases durability and flexibility of the device.
- **Transport of cryopreserved pathogens**: The removal or addition of materials from the cryopreserved inventory must be carefully documented, including full information on transfer of custody. Whether transported by courier or moved across the building, cryopreserved materials should be properly packaged and labeled with biohazard symbols to prevent accidental exposure to others and potential release to environment. *Cryptosporidium*, for example, is classified by the DOT as a Category B biological hazard, thus shipping should follow UN3373, Class 6.2 regulations.[72] Given the complexity of these guidelines, institutions require specific training courses that provide up-to-date requirements for certification to ship biological materials.
- **Maintenance of SOPs over time**: The prolonged storage of pathogens is vulnerable to a lapse in oversight due to personnel change or facility closures. Such a lapse may pose a risk to biosafety, biocontainment, and biosecurity. Establishment and monitoring the adherence to SOPs for storage of pathogens can guarantee continuity. Particularly critical is the SOP for reporting potential exposures and breaches to oversight authorities for risk assessment and mitigation. As long as pathogens remain in biorepositories, IBCs or relevant bodies should maintain oversight and ensure compliance with policies, which may evolve over time. Additionally, pathogens may lose viability over time, depending on the temperature of long-term storage. Implementation of periodical viability testing is therefore essential to ensure consistency of the inventory and inform the need for renewal of stocks.

## Conclusion

Advanced cryopreservation technologies hold enormous potential to facilitate much needed research on pathogens. However, cryopreservation will also extend the geographical and temporal reach of those pathogens as they are transported and stored, in some cases indefinitely. Indeed, the risk posed by inadvertent or malicious release of pathogens may alter over storage period — for instance, if population vulnerability to the pathogen has increased or decreased.

Too little attention has been paid to the benefits and risks of cryopreserving pathogens, including those like *Cryptosporidium* that have resisted conventional cryopreservation but now are being successfully preserved using novel techniques such as vitrification. By analyzing two BSL-2 pathogens that pose different risks, we suggest the type of analysis that needs to be developed going forward in a new era of advanced cryopreservation.

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Justyna Jaskiewicz, Rebecca D. Sandlin, and Mehmet Toner filed a patent protection for the vitrification cassette technology (US Patent App. 17/912,938). Additionally, Dr. Toner has patent applications relevant to tissue and organ preservation and has a financial interest in and serves on the Scientific Advisory Board for Sylvatica Biotech Inc., a company focused on developing high subzero organ preservation technology; his competing interests are managed by Massachusetts General Hospital and Mass General Brigham in accordance with their conflict-of-interest policies. Susan M. Wolf has no relevant disclosures.

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