Development and characterization of animal models for monkeypox virus infection

by

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Abstract

Monkeypox virus (MPXV), a member of the genus *Orthopoxvirus*, replicates mainly in rodents and other small mammals with occasional spillover to other animals and humans. Historically, Mpox was primarily reported in endemic regions of Africa, where it predominantly affected children and was transmitted primarily through direct contact with infected wild animals. The 2022 global clade IIb outbreak, with more than 100,000 cases reported across over 100 countries, demonstrated the spread of MPXV well beyond endemic regions. In contrast to endemic Mpox, the 2022 outbreak was characterized by generally milder disease and efficient human-to-human transmission, with men, particularly men who have sex with men (MSM), being the most affected group.

The global dissemination of MPXV clade IIb has raised concerns about potential spillover into susceptible animal populations, subsequent viral adaptation, and the establishment of novel animal reservoirs. The susceptibility of cattle, which are closely associated with humans and susceptible to related orthopoxviruses, to MPXV remains unknown. Here, we exposed calves to MPXV clade IIb simultaneously via intranasal, intradermal, and intravenous routes. Animals remained subclinical, with no overt signs of Mpox. Low levels of viral DNA were detected in nasal and oral swabs from a subset of experimentally infected animals, with infectious virus recovered from nasal swabs at early time points. Viral DNA was not detected in internal organs and lymph nodes, suggesting transient, localized viral replication without systemic dissemination. These findings indicate that cattle exhibit limited susceptibility to MPXV clade IIb, suggesting that sustained infection and the establishment of cattle as a reservoir are unlikely under the experimental conditions evaluated.

Human-to-human transmission of Mpox was historically limited; however, during the 2022 outbreak, intimate sexual contact, particularly within MSM networks, emerged as the predominant transmission route. Experimental studies investigating MPXV infection via rectal and genital mucosal routes remain limited, highlighting the need for suitable small animal models to better characterize MPXV infection and pathogenesis following mucosal exposure. Therefore, we infected CAST/EiJ mice with MPXV clade IIb via intraperitoneal or mucosal (intranasal, rectal, and genital) routes to evaluate route-dependent susceptibility, clinical outcomes, and viral shedding. Intraperitoneal infection resulted in moderate, non-lethal disease, particularly in male mice, which exhibited mild body weight loss accompanied by elevated clinical scores; viral DNA was detected in oropharyngeal swabs and tissues collected at necropsy. In contrast, mucosal inoculation via intranasal, rectal, or genital routes did not induce overt clinical signs, as mice remained largely asymptomatic, with no skin lesions and minimal to no body weight loss. Nonetheless, viral DNA was consistently detected in oropharyngeal, rectal, and genital swabs following inoculation via the respective mucosal routes. This sustained detection of viral DNA at the mucosal inoculation sites is consistent with localized viral replication in the associated mucosal tissues, although detection of viral DNA alone does not confirm productive infection. Together, these findings suggest that CAST/EiJ mice could serve as a non-lethal model for investigating mucosal MPXV infection and pathogenesis, potentially recapitulating aspects of the mild, self-limiting disease observed during the 2022 outbreak in contrast to conventional lethal challenge models. Further characterization of this model is warranted, particularly to confirm productive viral replication and assess its potential for virus transmission.

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Dedication

To my parents, who always encouraged me to dream big,

and to all the people, places, and moments that inspired me towards research.

Chapter 1 - Introduction

Mpox and Monkeypox Virus

The genus *Orthopoxvirus* belongs to the subfamily *Chordopoxvirinae* within the family *Poxviridae*. It comprises 17 recognized species with a broad host tropism and is divided into two major groups: Eurasian/African orthopoxviruses and North American orthopoxviruses. While some species such as variola virus of humans and ectromelia virus of mice are heavily host-restricted, others are capable of infecting multiple species. The natural reservoirs of most orthopoxviruses remain unknown. The genus includes several highly significant viruses, many of which are causative agents of severe disease and are zoonotic in nature, with documented outbreaks in human populations[1–5]. Variola virus is the causative agent of smallpox, a historically devastating disease that was eradicated worldwide in 1980 following a global vaccination campaign. The smallpox vaccine, developed using vaccinia virus, also conferred cross-protective immunity against other orthopoxviruses[6,7]. However, following cessation of routine vaccination after successful eradication, an expanding pool of unvaccinated individuals, comprising all birth cohorts since 1980, has become susceptible to infection by other orthopoxviruses. This shift has coincided with the emergence and re-emergence of several orthopoxviruses in recent years, including Monkeypox Virus (MPXV)[7–10].

Mpox, also known as monkeypox, is a zoonotic disease caused by monkeypox virus (MPXV), a member of the *Orthopoxvirus* genus. Monkeypox virus was first identified in 1958 in a colony of captive cynomolgus macaques at the Statens Serum Institut in Copenhagen, Denmark, following outbreaks of a smallpox-like vesicular rash illness in animals imported from Singapore[11]. Although its definitive natural reservoir remains unknown, the disease primarily circulates among rodents and small mammals in endemic regions of Africa, with sporadic

spillover to humans[12,13]. Since the first reported human case in 1970, mpox has remained endemic in Central and West African countries, causing outbreaks in small clusters that primarily affect children[14,15]. In recent years, however, large-scale outbreaks within endemic regions and multi-country epidemics from global dissemination of the virus have once again drawn worldwide attention to orthopoxvirus diseases. This global spread of mpox has prompted the WHO to declare Public Health Emergencies of International Concern (PHEICs) twice: once for the MPXV clade IIb outbreak in 2022, and again for the MPXV clade Ib outbreak in 2024, each driven by newly emerged viral lineage [16–18].

Monkeypox virus

Virion structure

Monkeypox virus (MPXV), like other members of the poxvirus family, is among the largest known animal viruses, with virion sizes ranging from 220–450 nm in length and 140–260 nm in width. Morphologically, a mature MPXV virion is brick- or ovoid-shaped and comprises four major structures, from innermost to outermost: the nucleoprotein core, lateral bodies, outer membrane and outer lipoprotein envelope (Figure 1.1). The nucleoprotein core is biconcave, dumbbell-shaped, housing the double-stranded DNA genome and associated fibrils. Flanking both concave sides of the core are the lateral bodies, which are further encased by an outer membrane surfaced with tubular proteins[19,20].

Mature MPXV virions exist in different forms with respect to their outer lipoprotein envelope and these forms are thought to serve different roles in dissemination within host and transmission between the hosts. A mature virion with a single bilipid membrane located in the cytoplasm is termed an intracellular mature virus (IMV). When the IMV acquires an additional

double layer of membrane derived from the trans-Golgi network or endosomes, it forms a wrapped virion, referred to as intracellular enveloped virus (IEV). Upon externalization of IEV via fusion with the cell membrane, the outermost membrane layer is lost, yielding extracellular virions (EV). EVs that remain associated with the cell surface are termed cell-associated enveloped viruses (CEV), whereas those released into the extracellular medium are called extracellular enveloped viruses (EEV). IMV and EEV are the two predominant infectious particle forms of MPXV: IMVs are released during cell lysis and possess a stable lipoprotein envelope suited to persistence in the environment and transmission between hosts, while EEVs are released via budding or exocytosis and mediate dissemination within the host[2,21].

Viral genome

The MPXV genome is double-stranded DNA with covalently closed hairpin ends. Like other poxviruses, the genome is large and complex, ranging from 196 to 211 kilobase pairs (kbp), with clade I showing more uniform genome lengths (196–199 kbp) across isolates than clade II (196–211 kbp). More than 190 open reading frames (ORFs) encoding proteins of over 60 amino acid residues have been identified. The genome consists of a central region that is tightly packed with orthopoxvirus genes (OPGs), which are conserved across orthopoxviruses and encode housekeeping proteins involved in transcription, RNA processing, replication and virion assembly (Figure 1.1). Flanking this central core region are variable terminal regions, which encode proteins involved in determining host range, disease pathogenesis and immunomodulation. Most proteins encoded by these regions are dispensable for *in vitro* replication; however, their absence results in viral attenuation in infection models, and they are therefore referred to as virulence factors. Each terminal region ends in an oppositely oriented,

identical inverted terminal repeats (ITRs) containing multiple tandem repeats, which terminate in the covalently closed hairpin loops that give the genome its dumbbell shape.[22–25].

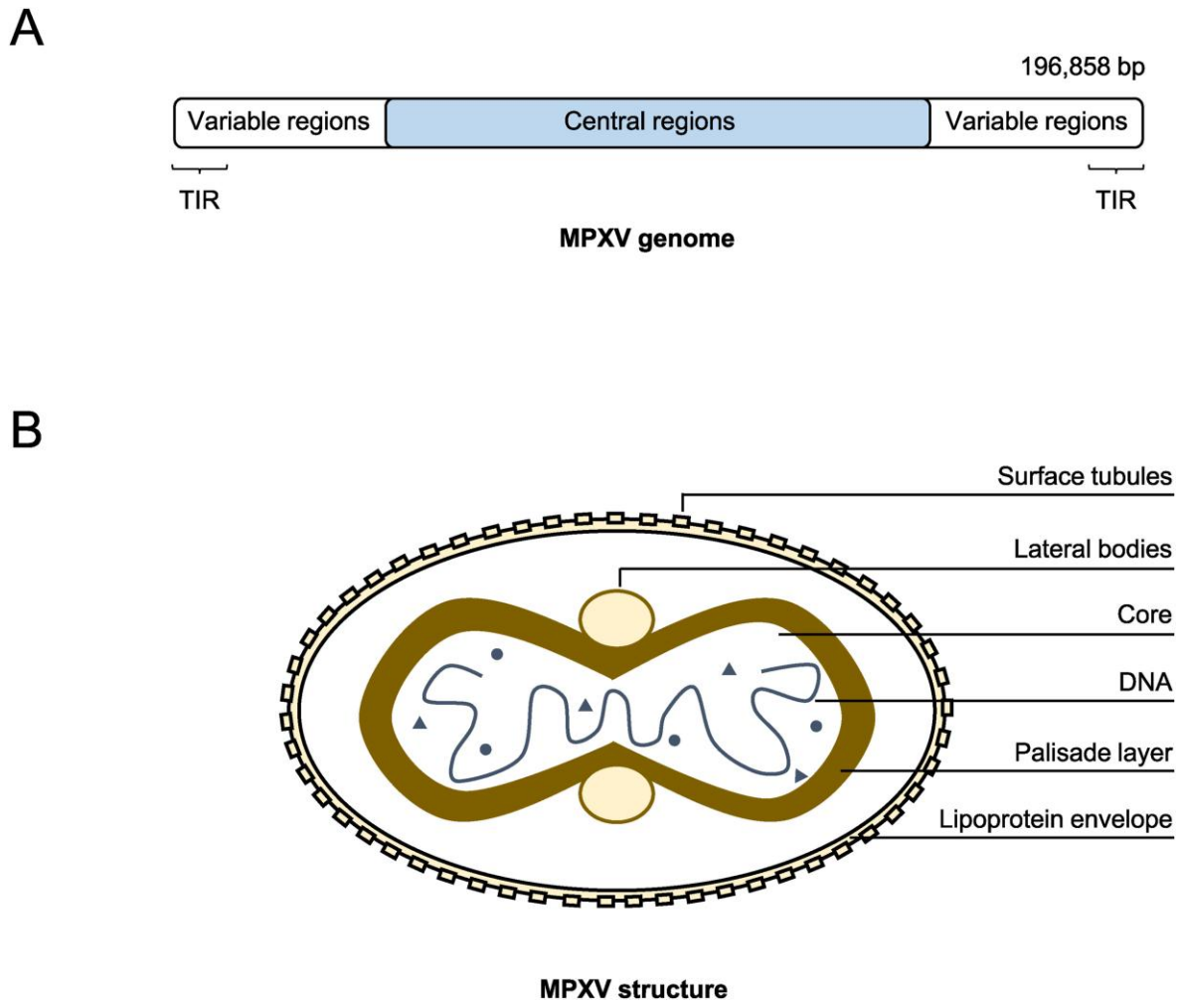


Figure 1.1 MPXV genome (A) and structure (B). Reproduced without modification from Yi X-M, Lei Y-L, Li M, Zhong L, Li S. The monkeypox virus–host interplays. Cell Insight 2024;3(5):100185. https://doi.org/10.1016/j.cellin.2024.100185 . Licensed under CC BY-NC-ND 4.0 (https://creativecommons.org/licenses/by-nc-nd/4.0/).

Virus replication

Unlike most DNA viruses, MPXV replicates entirely in the cytoplasm, specifically at discrete sites known as viral factories or Guarnieri bodies, where transcription, translation, and viral assembly take place. The replication cycle is similar to that of other orthopoxviruses. MPXV enters the host cell through attachment of viral surface proteins to cell surface receptors, aided by molecules such as chondroitin sulfate, heparan sulfate, laminin, and other cellular attachment factors, with entry occurring via either membrane fusion or endocytosis. Several MPXV surface proteins, including E8, A29, H3, A21, and A16, are involved in host cell entry. After entry, the virus uncoats, releasing the viral core particle into the cytoplasm. Cellular defenses are then inactivated and early genes are transcribed and translated, producing proteins that facilitate further uncoating, DNA replication, and the production of intermediate transcription factors. These intermediate factors drive expression of late genes encoding structural and non-structural proteins, which together with concatemeric DNA are packaged to form intracellular mature virions (IMV), released during cell lysis. IMV can acquire an additional double membrane derived from the trans-Golgi network or endosomes and, upon externalization, exist as cell-associated enveloped viruses (CEV) or extracellular enveloped viruses (EEV), as described above.

Mpox in humans

Clades of MPXV

MPXV clade I and clade II represent distinct genetic lineages of MPXV circulating in endemic regions of Africa. Clade I was previously known as the Central African clade or Congo Basin clade, owing to its prevalence in Central Africa, particularly in the Democratic Republic of

the Congo (formerly known as Zaire). Clade II, similarly, was known as the West African clade, named after the region of its predominant circulation[26]. Beginning in May 2022, a global outbreak of mpox occurred, caused by a newly emerged strain of MPXV that spread rapidly across multiple continents beyond its traditionally endemic range. Genomic surveillance revealed that this outbreak strain was phylogenetically distinct from viruses predominantly circulating in endemic regions[27]. In August 2022, WHO convened an ad hoc expert meeting to review the phylogeny and characteristics of MPXV clades and to propose a revised, consensus-based nomenclature. The meeting recommended using Roman numerals to denote clades, with lowercase Latin letters for subclades. Under this new system, the Congo Basin clade became clade I and the West African clade became clade II, each comprising its own distinct subclades. The strain responsible for the 2022 outbreak was designated clade IIb[28]. Subclades IIa and IIb are phylogenetically distinct, having evolved separately from a common ancestor over an extended period. Although clade IIb circulated extensively in recent years, particularly during the 2022 global outbreak, it has in fact been in circulation since the 1970s[23,28]. All subclades exhibit characteristic epidemiological features and remain genetically divergent from one another.

Genomically, the two major MPXV clades differ by approximately 0.5% of their nucleotide sequences. The central region is highly conserved across clades, whereas the terminal regions, which encode many proteins involved in host tropism, immune modulation and virulence, exhibit greater variability[29,30]. Clade II lacks an approximately 10 kbp region present in clade I, resulting in loss of the virulence gene D14L (OPG32). This gene encodes a complement-binding protein known as the monkeypox inhibitor of complement enzymes (MOPICE), which inhibits complement-mediated antiviral responses. Similarly, OPG195 (B14R), encoding an IL-1

receptor homologue involved in suppressing inflammatory responses, is truncated or absent in both clades IIa and IIb[29,31,32]. Further differences distinguish clade IIa from clade IIb: OPG165 (N3R), encoding an MHC class I homologue, and OPG005 (N1R), encoding an anti-apoptotic BCL-2 domain homologue, are present in clade IIa but absent in clade IIb, while genes encoding a type I interferon decoy receptor and an inhibitor of apoptosis carry mutations in clade IIb that may contribute to differences in virulence[23,29,31,33]. In the recently emerged lineages of clades IIb and Ib, APOBEC3 signature mutations have accumulated across both coding and non-coding regions of the genome, consistent with sustained human-to-human transmission[34]. Comprehensive comparative analyses of gene content, amino acid substitutions, insertions, deletions and other genomic variations are therefore necessary to fully elucidate the genetic determinants underlying the phenotypic differences among MPXV clades[29–33,35].

Clade Ia, formerly known as the Congo Basin strain, circulates in endemic regions of Central African countries, including the DRC, Republic of the Congo, Sudan, Cameroon, Central African Republic and Gabon. Sporadic and small-scale outbreaks of clade Ia have been recorded in the region since its first detection in the DRC in 1970. Clade Ia is the most virulent of all MPXV variants, causing severe disease with a case fatality rate of up to 10%[36,26,37]. In clade I, immunomodulatory genes are greater in number, more conserved, and more transcriptionally active compared to other clade, contributing to enhanced immune evasion, greater systemic dissemination and consequently increased disease severity[32]. Children, aged 15 years and younger, are the most affected demographic. Infection is typically acquired through zoonotic spillover from wildlife, usually via handling of infected animals or through bites and scratches from infected animals[37]. Clade Ia continues to circulate in Central African countries[38].

Clade Ib is the most recently emerged subclade of clade I, responsible for outbreaks in the Central African endemic region since 2023. This strain is both genetically and epidemiologically divergent from clade Ia, which has historically circulated in the region. Like clade IIb, which caused the 2022 global outbreak, clade Ib has accumulated APOBEC3 (apolipoprotein B mRNA editing enzyme catalytic polypeptide 3)-mediated signature mutations and exhibits sustained human-to-human transmission driven by intimate sexual contact[39,40]. Unlike clade Ia, which primarily affects children, the major demographic affected by clade Ib is young men (aged 15–35 years). The clinical presentation closely resembles that of clade IIb infections, with anogenital lesions and associated complications being the most prevalent features; however, disease severity tends to be greater than that observed during the 2022 outbreak[39,41].

Clade IIa is endemic in West African countries, including Nigeria, Ivory Coast, Sierra Leone, and Liberia[37]. This strain has undergone minimal genomic change over time and has remained relatively stable throughout its evolutionary history[26]. Compared to clade I, clade IIa causes less severe disease, presenting with moderate clinical signs and a lower case fatality rate of up to 3%. The virus also circulates among wild rodents and small mammals in rural rainforest habitats, with transmission dynamics similar to those of endemic clade I[26]. The first human mpox outbreak outside Africa occurred in the United States in 2003 and was caused by clade IIa following the importation of infected rodents from Ghana[42].

Clade IIb, known to have circulated since the 1970s, has undergone several evolutionary changes over time, including gene loss and accumulation of mutations, predominantly APOBEC3-driven substitutions in recent years [20,23]. The newly emerged lineage of clade IIb was responsible for the 2022 global outbreak of mpox, which spread to more than 100 countries with

over 100,000 reported cases. The shifting epidemiology, altered transmission dynamics, and atypical clinical presentation observed during this outbreak have drawn considerable scientific attention to clade IIb, prompting extensive research into the virus and the disease it caused[43]. The clade IIb outbreak and its epidemiology are discussed in detail in a later subchapter.

Transmission

MPXV can be transmitted through direct or indirect contact with infectious viral particles originating from animals, humans or the environment. Zoonotic transmission typically occurs through contact with infected animals during hunting, handling, skinning, butchering and carcass preparation, as well as through bites and scratches from infected animals or consumption of contaminated bushmeat[44]. Veterinarians handling infected animals may likewise acquire MPXV infection[45]. Human-to-animal transmission is uncommon but was recently reported in a pet dog in France[46].

Human-to-human transmission can occur through direct contact, respiratory droplets, or indirect contact with contaminated materials. Prior to the 2022 outbreak, indirect contact with infectious particles from lesions or bodily fluids and aerosol transmission via respiratory droplets were considered the major routes of transmission[37,47]. However, with the efficient human-to-human transmission observed during the 2022 clade IIb outbreak, direct skin-to-skin contact, whether through sexual or other forms of intimate contact, emerged as the predominant route driving the global outbreak within men having sex with men (MSM) communities. During this outbreak, a high viral burden was detected in semen, saliva, and rectal and oropharyngeal swabs[48–50]. Contaminated surfaces and objects can also serve as a source of infection as the virus

can remain viable for up to 15 days[48,51]. Vertical transmission from infected mother to newborn has been well documented in humans and demonstrated experimentally in animal models[52].

Pathogenesis and clinical signs

MPXV can enter the host via the mucosal membranes of the oral cavity, respiratory tract, rectum, and genital tract, or through breaks in damaged skin. The virus initially replicates in mucosal cells, keratinocytes, fibroblasts and endothelial cells. It is then taken up by nearby tissue-resident immune cells, including monocytes, macrophages, and dendritic cells, which subsequently migrate to the draining lymph nodes. The virus replicates extensively within lymphoid tissues before disseminating via the lymphohematogenous route to other organs, such as the spleen, liver, and tonsils. Replication in these organs, followed by release of viral particles into the bloodstream, leads to a secondary viremia, which drives widespread dissemination of the virus to additional organs, including the lungs, kidneys, and reproductive tract. Generalized rash appears once the virus reaches the skin, disseminated via dermal blood vessels and carried by migrating immune cells such as dendritic cells and macrophages[53,15,54].

The severity and clinical features of mpox are influenced by the infecting virus strain, transmission route, and host factors such as immune status and comorbidities. Although the majority of clinical signs are common across outbreaks caused by all clades, their extent, presentation, and severity vary depending on the factors mentioned above[44]. For example, rash and lymphadenopathy are the most common and consistently reported findings in human mpox; however, both were more generalized in historical endemic mpox compared to the more regional or localized presentation seen in recent outbreaks driven predominantly by sexual contact[55–58]. The incubation period of mpox ranges from 5 to 21 days, with an average of 5 to 8 days. Non-

specific prodromal signs, including fever, chills, muscle pain, and lymphadenopathy, typically precede the rash by 2 to 3 days. The generalized rash classically begins on the head and extremities before spreading across the body[44,59]. In recent outbreaks, however, prodromal signs were less frequently reported, or occurred concurrently with or after the onset of rash. Rash was predominantly localized to the anogenital region and, in some cases, presented solitarily in the region, clinical features most likely attributable to the sexual contact route of transmission[55,57,58]. Proctitis emerged as another novel, atypical clinical sign associated with sexual transmission[60,61]. In typical mpox, the rash progresses from papules to vesicles, then pustules, ultimately forming crusts and scars, a process usually taking 2 to 4 weeks. During the 2022 outbreak, however, this staged progression was not consistently observed[54,55].

Vaccines and antivirals

Two vaccines are approved by the FDA for the prevention and control of mpox: an attenuated third-generation vaccine based on modified vaccinia Ankara (MVA) (marketed as JYNNEOS/IMVANEX/IMVAMUNE), and a second-generation live VACV vaccine, ACAM2000. Both were originally developed against smallpox. The MVA vaccine is administered as two doses given intradermally 28 days apart and offers 66–90% protection following the second dose. It has a more favorable safety profile than ACAM2000. ACAM2000 is a fully replicating second-generation vaccine that provides strong immunogenicity but is associated with adverse effects, including myocarditis and pericarditis[62,63]. Dryvax, the first-generation live VACV vaccine from which ACAM2000 was derived, was reported to be approximately 85% effective against MPXV infection. The mpox indication for ACAM2000 was

based on these historical data and on challenge studies in macaques rather than on human efficacy trials[64,65].

Antivirals originally effective against smallpox are currently used as therapeutics for mpox, though clinical data on their efficacy against MPXV remain limited. The two most widely used antivirals approved by FDA for mpox treatment are tecovirimat and brincidofovir, although brincidofovir is not approved for this indication in Europe by the European Medicines Agency. Tecovirimat targets the viral VP37 membrane protein, an ortholog of vaccinia virus F13 protein, preventing viral envelope formation and the release of extracellular virions, thereby disrupting viral spread; however, it is not effective against the intracellular form of the virus. Brincidofovir inhibits viral DNA polymerase-mediated DNA synthesis, but its clinical application is limited by hepatotoxicity[66,67].

Epidemiology and global emergence of human Mpox

Endemic mpox in Africa: historical epidemiology

Historically, mpox was restricted to endemic regions of Africa. For five decades following its initial detection, confirmed cases of mpox, except rare exportations, were not reported outside west and central Africa. The first documented case of mpox in humans was in a 9-month-old boy from the equatorial region of the Democratic Republic of the Congo (DRC) in 1970. The child presented with smallpox-like clinical symptoms, which was later confirmed by WHO collaborating centers on smallpox and other poxvirus infections in Atlanta and Moscow to be caused by monkeypox virus [68,69]. Between 1970 and 1979, 48 confirmed cases of mpox were reported across six African countries: the DRC (n =38), Cameroon (n =1), Côte d'Ivoire (n =1), Liberia (n =4), Nigeria (n =3), and Sierra Leone (n =1). In endemic regions, disease outbreaks were primarily linked to direct contact with infected animals through activities such as hunting, skin preparation and consumption of meat of infected rodents and primates[68,69].

Smallpox, another lethal orthopoxvirus disease, was eradicated globally around the same time through mass vaccination campaigns using the vaccinia virus; the last indigenous case of smallpox was documented in Somalia in October 1977[70]. Because orthopoxviruses share high sequence homology and structural similarity, the vaccinia vaccine provided cross protection against mpox[71]. However, the Global Commission for the Certification of Smallpox Eradication deemed continued vaccinia vaccination unnecessary for mpox control, as sporadic cases did not represent a significant public health threat. Nevertheless, the commission recommended investigation of the public health importance of mpox, which prompted serological surveys of unvaccinated children in endemic mpox regions in 1979. These surveys reported orthopoxvirus

seropositivity in 15.4% of 10,300 samples. Among the 420 orthopoxvirus-positive sera, 73 had monkeypox virus-specific antibodies[68].

Following the serological survey results and the commission's recommendation, the World Health Organization (WHO) supported the establishment of an intensive active surveillance program for human mpox in the DRC from 1981 to 1986. During this period, 338 confirmed human mpox cases were identified, with the majority occurring in rainforest regions of the country. Most cases (86%) occurred in children younger than 10 years of age and the case fatality rate was approximately 9.8%. Direct contact with infected animals was identified as the primary source of infection[68].

Between 1987 and 1992, only 13 cases were documented in west and central Africa, predominantly in children, likely reflecting a reduction in surveillance. No further cases were reported globally until 1996, when the DRC experienced a major resurgence. Epidemiological investigations into the 1996–1997 DRC outbreak identified over 300 cases across multiple villages, with children remaining the primary affected demographic[68,72–74].

First mpox exportation to the Western Hemisphere: the 2003 USA outbreak

The first mpox disease outbreak outside Africa was recorded in 2003 in the United States of America, also referred to as "The 2003 Midwest Monkeypox Outbreak." On May 24, 2003, a 3-year-old girl was hospitalized after being bitten by a prairie dog purchased at a swap meet in Wisconsin; she presented with fever and cellulitis. Similarly, on June 2, nodular skin lesions were reported in a person working for an exotic animal distributor in Wisconsin. Following epidemiological investigation, contact tracing and diagnostic testing by the Wisconsin Division of Public Health and the CDC, mpox was confirmed as the causative disease for above cases as

well as other cases linked with prairie dogs. Prairie dogs were positive for MPXV-specific DNA by PCR. Further investigation revealed that the prairie dogs had been housed in proximity to infected rodents of different species that were imported from Ghana, establishing the source of the outbreak[75–77].

In total, 47 cases (37 confirmed, 10 probable) were reported across Illinois, Indiana, Kansas, Missouri, Ohio and Wisconsin, transmitted through direct or indirect contact with MPXV-infected animals. Most cases were attributed to direct exposure through contact with infected pets, while the remaining cases were associated with exposure in pet stores, animal swap meets and veterinary clinics. Notably, no person-to-person transmission or human deaths were documented in this outbreak. Patients with bite or scratch wounds from infected animals exhibited more severe systemic illness signs compared to those with non-invasive exposures such as respiratory or mucosal contact. Common clinical symptoms included fever, chills, lymphadenopathy, sweats, headache, myalgia and sore throat[76,78].

The 2003 mpox outbreak in the United States demonstrated that translocation of animal species between geographic regions can facilitate disease emergence in previously unaffected areas. In response, the CDC and FDA jointly implemented a ban on rodent imports from Africa and imposed restrictions on the sale, distribution and transport of prairie dogs to prevent further spread of MPXV[79].

Re-emergence in Nigeria: the 2017 outbreak

The first reports of mpox in Nigeria date back to 1971. On April 4, 1971, a 4-year-old girl living in south-eastern Nigeria who presented with febrile illness and vesiculopustular skin lesions was confirmed as the first laboratory-confirmed case of mpox in Nigeria. Nine days later,

her mother developed fever and a skin rash and is suspected as the first documented instance of human-to-human mpox transmission, although definitive evidence is lacking. Between 1971 and 1978, 10 mpox cases were reported in Nigeria[14,80]. These sporadic cases and small outbreak clusters occurred predominantly in rainforest areas, with the majority observed in rural communities surrounding freshwater swamps and mangrove habitats[81].

In 2017, Nigeria experienced a resurgence of mpox outbreaks after 39 years without human mpox reports. On September 23, 2017, an 11-year-old boy hospitalized with suspected atypical chickenpox was confirmed to have mpox following positive PCR detection of MPXV in a skin swab. In the following months, the number of reported mpox cases increased rapidly across the country[82].

Between September 11, 2017 and September 16, 2018, a total of 276 suspected human mpox cases were identified, with the majority reported during the last quarter of 2017. Of these, 114 cases were laboratory confirmed by PCR and/or detection of anti-MPXV IgM antibodies. All confirmed patients presented with vesiculopustular rash. Fever, headache, pruritus, lymphadenopathy and chills were among the most frequently reported clinical symptoms. Seven deaths were reported, four of which occurred in individuals with underlying HIV/AIDS[81,83].

In contrast to the historical epidemiology of mpox, where the disease predominantly affected rural communities in rainforest regions with children as the primary age group affected, the 2017 outbreaks showed a shift in epidemiological patterns: a substantial proportion of cases occurred in urban communities and young adult males represented the most affected demographic. Genomic and epidemiological investigations suggested multiple independent introductions of the virus from animal reservoirs into different human communities and human-to-human transmission in small clusters. The documentation of mpox cases among healthcare

workers and multiple infections within a prison facility further corroborates the person-to-person transmission during this outbreak[83].

The second incidence of mpox outside of Africa occurred in 2018, when cases were reported in the United Kingdom. Unlike the 2003 outbreak, the export of the disease was associated with the travel of infected person from the endemic regions. Between 2018 and 2021, a total of eight travel-associated and three secondary mpox cases were reported in the United Kingdom, the United States, Singapore and Israel. High-throughput sequencing confirmed that all of these travel-associated cases originated from Nigeria[21].

Global emergence: the 2022 multi-country outbreak

The 2022 global mpox outbreak is the largest in terms of magnitude and geographical spread ever recorded in mpox history. The sheer scale of the outbreak and affected regions caught the world off-guard at a time when global recovery from the SARS-CoV-2 pandemic was still underway. More than 110 countries on all inhabited continents were affected with a total of over 100,000 confirmed human mpox cases globally. The outbreak was caused by a newly emergent subclade IIb[84,85].

In early May 2022, multiple reports of human mpox cases began emerging in countries where the disease is not endemic and had not been previously reported (Figure 1.2). The first confirmed case was reported on May 6 in the United Kingdom in a British national who had returned from Nigeria and presented with mpox symptoms. Contact tracing was immediately initiated to identify potential secondary cases. On May 12, two additional mpox cases were identified in the same household in London. On May 17, four mpox cases were identified in

individuals self-identifying as gay or bisexual. These cases had no epidemiological link to the initial case and no travel history to endemic regions[86–88].

On May 17, the United States reported its first mpox case in a man hospitalized on May 12 with symptoms consistent with mpox and a recent travel history to Canada[89].

On May 18, Spain reported seven confirmed and 22 suspected mpox cases. The majority of cases were linked to a sauna regarded as a gay-friendly establishment[89,90]. On the same day, Portugal reported mpox in five men identified as MSM who had presented with genital lesions to sexually transmitted infection clinics[91].

On May 19, Sweden, Italy, Belgium, and Canada reported their first cases. On May 20, Australia reported two cases in individuals who had recently returned from Europe. On the same day, France, Germany and the Netherlands confirmed their first mpox cases. Early cases in the Netherlands were identified in MSM linked to a festival held in Belgium[89].

Over subsequent days, Switzerland, Israel, Austria, Denmark, the Czech Republic, the UAE, Slovenia, Argentina, Finland, Ireland, Mexico and numerous other countries reported cases. The majority of early cases in these countries were travel-associated, majority with links to travel within Spain or other European countries[69].

The Gran Canaria Pride Festival held from May 5–15, 2022, is considered as one of the origin points for mpox transmission across European countries. The festival had attendees of approximately 25,000–30,000 foreign visitors. Early cases detected in European countries were epidemiologically linked to travel to Gran Canaria during the festival. Gran Canaria reported one of the highest concentrations of mpox cases relative to its population density. Among mpox patients presenting to the Infectious Diseases and Tropical Medicine Unit in Gran Canaria, 88% were identified as MSM and over half were foreign visitors[92].

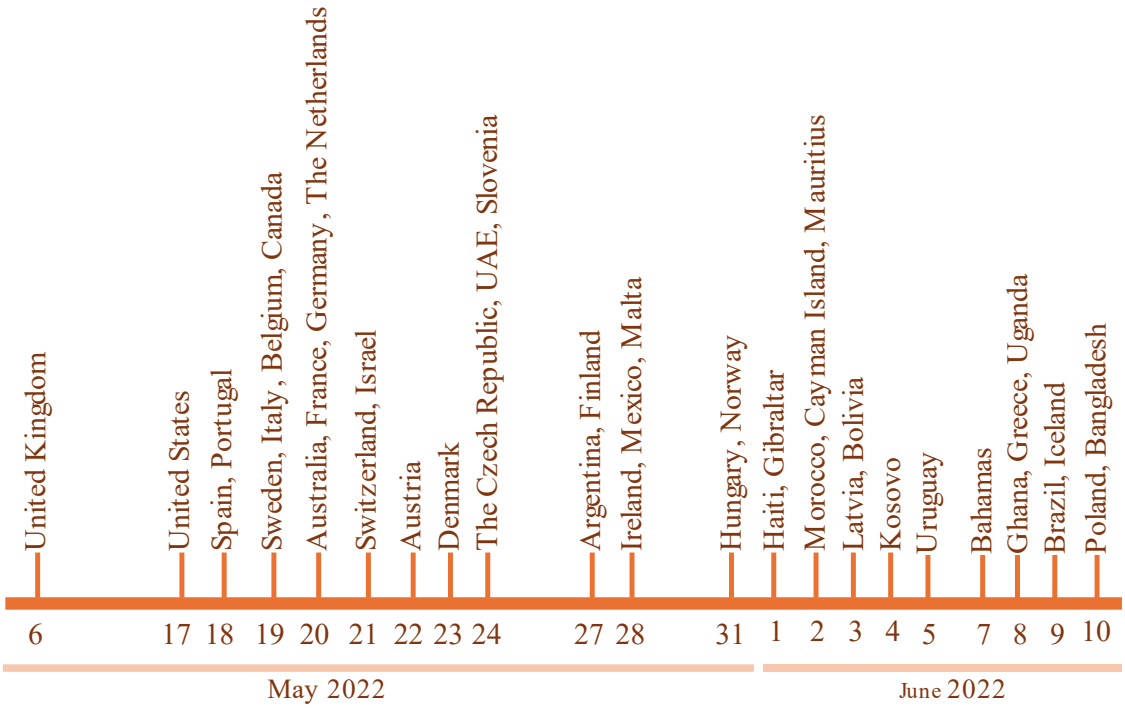


Figure 1.2 Chronology of first reported mpox cases by country during 2022 mpox outbreak[93,94]. Countries are plotted according to the date on which their first laboratory-confirmed cases of mpox were reported, from the initial case in the United Kingdom on 6 May 2022 through 10 June 2022. Intervals along the axis are not drawn to scale.

As human mpox cases were detected across multiple continents, countries with confirmed reports implemented active surveillance, contact tracing and control measures including self-isolation and quarantine, while unaffected countries began preparing public health measures and emergency preparedness plans to prevent mpox introduction and transmission. Belgium became

the first country to introduce compulsory 21-day quarantine for infected patients. The USA stockpiled smallpox vaccines (known as JYNNEOS) which are known to be effective against mpox. The UK offered post-exposure prophylaxis vaccination for close contacts[89].

With multiple countries reporting cases and case numbers rising, the WHO convened an Emergency Committee under the International Health Regulations to assess the global risk posed by the mpox outbreak and the WHO Director-General declared mpox a public health emergency of international concern on July 23, 2022. At that time, more than 16,000 confirmed human mpox cases had been reported to the WHO from 75 countries[18].

Case numbers continued to rise over the following months, peaking in July and August and declining toward the end of 2022. From January 1, 2022 to August 12, 2024, there were 99,176 mpox cases from 116 countries across all six WHO regions, with a total of 208 deaths, reported to the WHO. The Region of Americas and European regions reported the majority of cases, with the highest numbers reported from the United States (33,191), Brazil (11,212), Spain (8,084), France (4,272), Colombia (4,249), Mexico (4,124), the United Kingdom (3,952), Peru (3,875), Germany (3,857), and the Democratic Republic of the Congo (2,999). Together, these ten countries accounted for more than 80% of global mpox cases[95].

Historically, mpox outbreaks in endemic regions resulted from zoonotic spillover of the virus into rural human communities through direct contact with infected animals or consumption of bushmeat[96]. Sustained human-to-human transmission was not documented and the longest documented chain of human-to-human transmission comprised of six individuals[21,97]. In stark contrast, the 2022 outbreak was characterized by clusters of cases in urban centers and was predominantly driven by human-to-human transmission, primarily through intimate sexual contact in men who have sex with men (MSM) communities. The most commonly reported

transmission settings included small social gatherings with sexual contact, such as nightclubs, private parties and saunas. Early cases in the 2022 outbreak were associated with festivals attended predominantly by MSM. Transmission in household settings and large public events represented only a small proportion of cases[57]. These patterns indicate that intimate sexual contact, particularly within densely connected sexual networks among MSM, was central to the sustainment of the 2022 outbreak.

Of cases with available data from the 2022 global mpox outbreak, 96.4% of confirmed cases were male with a median age of 34 years. This predominance of cases in young adult males is in contrast with historical mpox epidemiology, which affected children regardless of gender in rural communities. Among these men, the majority (more than 80%) identified themselves as MSM, including gay, bisexual and other MSMs. Children represented only 1.1% of total cases in the global outbreak[57,95].

Clinically, the disease was less severe compared to previous outbreaks caused by the same clade as well as other clades. Clade I, responsible for outbreaks in Central Africa, has a case fatality rate (CFR) up to 10%, while clade IIa, responsible for historical outbreaks in West Africa, has a CFR of up to 3%[98]. In the 2022 outbreak caused by the newly emerged clade IIb, the CFR was 0.1%. Hospitalization and deaths were higher in immunocompromised and HIV-positive individuals. Only a smaller proportion of cases reported clinical symptoms during the prodromal phase[57,99].

The clinical presentation observed during the 2022 outbreak differed from prior documented cases of mpox. Rash is the most commonly reported symptom in mpox, and in this outbreak as well, rash was the most common symptom in patients, followed by fever, skin or mucosal lesions, genital rash, and headache. Earlier, rash and lesions were distributed throughout

the body, with the most concentration in the extremities and face; however, in this outbreak, generalized rash was less frequently reported, and lesions were mostly localized to the anogenital and oropharyngeal regions. In some cases, lesions were confined solely to the anogenital region, which is in stark contrast from earlier outbreaks. In typical mpox disease, the rash progresses sequentially through macules, papules, vesicles, pustules and umbilication, eventually falls off after crusting; however, in 2022 outbreak, this typical progression was not consistently followed[57,100–104]. The extent to which these atypical clinical features are attributable to novel route of viral inoculation (via sexual intimate contact), to genetically evolved strain, or to a combination of both, still remains to be elucidated. Cases presenting atypical clinical signs and symptoms that did not meet the existing WHO mpox case definition complicated the diagnosis and resulted in undetected cases[105–109]. Maruotti et al. estimated that the number of undetected mpox cases exceeded twice the number of reported cases during this outbreak[110]. There are also evidence of asymptomatic mpox infections[111–113], raising concerns about undetected transmission. Consequently, in an effort to reduce undetected cases and misdiagnoses, the WHO and various national health agencies updated the suspected mpox case definition to also include patients presenting with a single skin lesion, with lymphadenopathy in the absence of skin lesions, with mucosal lesions such as proctitis, or with prodromal symptoms following contact with an infected individual[105,114–117]. In this outbreak, viral shedding was highest in skin lesions, saliva, and anorectal and oropharyngeal swabs, with lower-level detection in semen, urine, and blood [57,104,118]. Rapid diagnostic and home-based testing kits employing these sample types could facilitate earlier detection and timely isolation, thereby helping to limit onward transmission.

Phylogenetically, the 2022 outbreak MPXV strains form a tight cluster that diverges as a separate, descendent branch from clade IIb strains circulating in Nigeria during 2018–2019[27]. Multiple genomic analyses of the 2022 outbreak strains have identified, on average, 42–50 single-nucleotide polymorphisms (SNPs)[27,34,119–122], which is a substantial number given the substitution rate normally expected for orthopoxviruses[34], suggesting accelerated viral evolution over a relatively short period. More than 80% of the substitutions identified are consistent with the action of host APOBEC3 editing on the viral genome[20,27,34,122,123], a frequency remarkably higher than that observed in endemic clades (8–13% in clades Ia and IIa)[34]. It is believed that the virus's susceptibility to APOBEC3 editing is not carried with its zoonotic spillover, but rather emerged following sustained replication within the human population[20,124]. APOBEC3 proteins are polynucleotide cytidine deaminases used by placental mammals as an antiviral defense mechanism; they introduce G-to-A and C-to-T mutations within single-stranded MPXV DNA intermediates, thereby inhibiting viral DNA replication. However, this APOBEC3-mediated antiviral process is inefficient at limiting MPXV replication, as the substitution rate it induces falls below the threshold required for effective viral inhibition[20,34,125]. Consequently, sustained human-to-human transmission drove the accelerated accumulation of APOBEC3 mutational signatures in the 2022 clade IIb outbreak strains[126,127]. APOBEC3 mutational signatures have also been observed in clade Ib, another newly emerged subclade responsible for outbreaks in the DRC since 2023, in which sustained human-to-human transmission is likewise well documented[128,129]. Sustained human-to-human transmission thus provided continued opportunities for APOBEC3-mediated mutagenesis, which together with natural selection, resulted in genetically divergent strains and may have contributed to the virus's adaptation to the human host and attenuated phenotype.

The 2022 mpox outbreak had unique, unprecedented epidemiological and clinical features that set it apart from endemic mpox. However, several critical questions surrounding this outbreak remain incompletely understood, including the underlying mechanisms of disease, the factors contributing to its novel transmission route and atypical clinical presentation, the effects of mutational changes associated with this emergent clade, the extent to which ongoing human adaptation may alter viral virulence and so on. Further studies integrating experimental, virological, immunological, genomic and epidemiological approaches are necessary to address these gaps, particularly as MPXV continues to circulate in new populations.

Animal models for Mpox research

Monkeypox virus (MPXV) has a wide host range with detection in species ranging from large nonhuman primates such as monkeys to small rodents like dormice. Our knowledge of MPXV host range continues to expand as new animal species are discovered to harbor the virus in the wild[130]. To date, however, the natural reservoirs of MPXV have not been definitively identified. Rodents and small mammals, particularly species found in endemic regions of Africa, are known to be naturally infected with the virus and are presumed to be the primary source of infection for other animals and humans[13]. Similarly, animal species in non-endemic regions can also be susceptible to MPXV, becoming infected when the virus is introduced to the region through imported infected animals or humans, as seen with infection of prairie dogs during 2003 USA outbreak[77]. This wide host range provides opportunities to investigate potential animal reservoirs and establish suitable animal models for studying mpox disease.

The scientific application of well-characterized animal models to study disease pathogenesis has been pivotal in advancing our understanding of infectious diseases and in the downstream development of diagnostics, drugs, and vaccines. Since early scientific history, the use of animal models, ranging from small rodents to large primates, has shaped the biomedical research into its current form[131,132]. Efforts to model mpox in animal systems began shortly after the virus was first isolated from animals in research facilities in Denmark[11]. Since then, numerous studies have been conducted using different animal models to investigate disease pathogenesis, immunology, transmission dynamics, and medical countermeasure development.

Nonhuman primates

Early experimental infections with MPXV were conducted in nonhuman primates (NHPs) following initial outbreaks of mpox in primate research facilities in Denmark (1958) and the USA (1959), where imported primates were housed for research purposes[11]. Experimental infection of MPXV in NHPs via different routes including intradermal (i.d.)[11,133], intravenous (i.v.)[133–144], intranasal (i.n.) exposure to aerosols[145,144,146,53,147,148], intramuscular (i.m.)[149,150], scarification[145], intratracheal[151,152], intrabronchial[153], and subcutaneous (s.c.)[133] resulted in characteristic skin eruptions (papules, pustules, vesicles) and virus recovery from skin lesions. Clinical signs included skin rashes and high fever. Most of these experiments conducted in the 1950s and 1960s utilized macaques (*Macaca mulatta and M. fascicularis*) because outbreaks of the virus occurred in these monkey species housed in captive facilities, demonstrating high susceptibility to MPXV when infected experimentally via multiple exposure routes[154]. Macaques have been used to study pathogenesis, vaccine, antiviral development, and transmission studies[136,154,155]. During the era of smallpox eradication, MPXV experiments in NHPs was geared towards developing a potential animal model for both smallpox and mpox[154,156,157]. The clinical signs in NHPs induced by virulent strains of MPXV, particularly clade I, are clinically similar to those of smallpox, with exception that lymphadenopathy is absent in smallpox infection[158].

There are several major advantages of using NHPs as models for MPXV. NHPs are, although rarely, naturally infected with MPXV in the wild and are thus highly susceptible to the virus[159]. The clinical signs and disease progression observed in NHPs closely resemble what is seen in human mpox, and the model best recapitulates the clinical disease progression, signs, and symptoms of MPXV infection[160–163]. NHPs, compared to other commonly used laboratory

animals, have close genetic, immunological, and physiological similarity to humans allowing for more reliable extrapolation of findings. Thus, NHPs offer a good model to evaluate vaccine and antiviral efficacy and effectiveness, as well as to study critical pathogenesis and host-viral interaction questions[164].

Studies in NHPs have been extensively conducted in Asian macaques instead of African primates which are naturally infected in the wild, as primates from Asian breeding facilities are easier to manage in research facilities[164]. Primates are large, wild animals that require careful management and handling compared to small, more docile rodents. Large facilities needed to house animals in high-containment conditions, limited availability of primates, high costs and ethical concerns are some of the reasons that research in NHPs is limited despite being a highly beneficial mpox model.

Small animal models

Small animal models such as mice, hamsters, and guinea pigs have been extensively used in experimental research of infectious diseases. They offer more opportunities and advantages compared to larger animals. Colonization and inbreeding of these rodents over time made them easily accessible. Commercial mouse vendors supply different strains of mice, while research laboratories maintain their own breeding colonies as needed. Rodents are easier in terms of housing and management, which makes them simpler to maintain, breed and use in research. Personnel training required to handle rodents are minimal compared to larger species such as NHPs. Widespread use of rodents in research has promoted development of reagents and research tools specifically targeted toward these species[165]. Accordingly, small animal models have been widely used in experimental mpox research.

Prairie dogs

In 2003, the USA recorded its first human mpox outbreak, which was linked to introduction of the virus into humans from prairie dogs (*Cynomys* spp.) that had been housed in close contact with various rodent species imported from Africa[42,75]. Infected prairie dogs died within a short period of time with clinical signs such as nasal and ocular discharge, oral ulcers and pulmonary consolidation[78,166]. The fact that MPXV is highly lethal in prairie dogs prompted exploration of prairie dogs as an option for animal modeling of mpox. Experimental infection in prairie dogs revealed that intraperitoneal and intranasal exposure of MPXV (clade I and IIa) is lethal at doses lower than those required in other animal models[167,168]. Using *in vivo* bioluminescent imaging, studies have also characterized viral kinetics and disease pathogenesis in this animal model[169,170]. The disease progression and presentation closely mirror those seen in human mpox. Transmission studies revealed that virus is readily transmitted to sentinel animals[171,172]. Prairie dogs have also been used in antiviral and vaccine studies[173,174]. Although an excellent model due to high susceptibility to both viral clades and close resemblance to the human systemic mpox, the inability to breed prairie dogs in captivity and the lack of available research reagents limit the utility of this model.

Squirrels

Monkeypox virus (MPXV) was first isolated in nature from Thomas's rope squirrel (*Funisciurus anerythrus*). Surveys in the 1980s revealed MPXV-specific antibodies in Thomas's rope squirrel, ribboned rope squirrels (*F. lemniscatus),* red-legged sun squirrels *(H. rufobrachium*) and Gambian sun squirrels *(H. gambianus*)[154]. Similarly, in the 2003 mpox

outbreak in the USA caused by import of infected rodents from Africa, transported squirrels also tested positive for MPXV DNA[42].

Studies with exposing the different squirrel species to mpox by Shelukhina et al. found that high-dose MPXV infection via intranasal, subcutaneous and per oral route resulted in acute, generalized infection with 100% lethality in *Funisciurus* sp., *Protoxerus* sp., and *Heliosciurus* sp[154]. Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) have been evaluated in several studies as a potential animal model. Intranasal and intraperitoneal infection of these squirrels with 10^5 PFU of MPXV clade IIa resulted in 100% lethality and high viral titers in liver, lungs and spleen; however, rash was not observed[175]. Infection of rope squirrels (*Funisciurus anerythrus*) with recombinant MPXV expressing firefly luciferase resulted in high lethality with oral lesions and heavy viral shedding[176]. Overall, squirrels are good reservoirs of the virus and their high susceptibility makes them a good candidate for lethality model of mpox to study systemic infections and evaluate vaccine and antivirals. However, several limitations constrain their use, including the difficulty of housing and breeding them in captivity in sufficient numbers, the lack of immunological reagents and their limited genetic characterization.

African dormouse

In the 2003 mpox outbreak in the USA, African dormice *(Graphiurus kelleni)* imported from Africa were suspected as possible shedders or transmitters of virus to prairie dogs[42]. An attempt to model this rodent for mpox studies revealed that vivarium-bred African dormice challenged with 10^4 PFU of clade I via intranasal infection resulted in 100% lethality with a very low LD_{50} of 12 PFU and high viral titers in blood and organs including spleen, liver and lungs[177]. The study also evaluated vaccination and antiviral efficacy against MPXV in this model[177].

Similarly, Americo et al. intranasally challenged 4–8-month-old African dormice with clade Ia, which resulted in 100% mortality at doses as low as 500 PFU with an LD_{50} of 12 PFU[178]. Other studies have reported similar results of high lethality following MPXV infection in dormice[179–181]. Although these studies report high susceptibility, MPXV-infected dormice did not develop the rash or cutaneous lesions characteristic of human mpox.

Mice

Mice (*Mus musculus*) are the most widely used animal models for studying the pathogenesis of infectious diseases in biomedical research[165,182]. Mice offer several advantages when compared to other rodents, such as commercial availability of well-characterized inbred and genetically modified strains, genetic homogeneity within inbred lines, larger variety of research reagents. Since the discovery of MPXV, there have been attempts to model the mouse species for experimental research to study pathogenesis, antiviral development and vaccine development. The earliest experimental report in mice with MPXV infection dates to 1960, two years after the discovery of MPXV, where researchers found that 3-week-old mice (strain not specified) were resistant to intraperitoneal injection but susceptible to intracerebral exposure. The same study found that suckling mice were susceptible to intranasal infection of MPXV resulting in death[133]. Other articles from that period also reported similar findings that mice were resistant to MPXV via the i.p. route but succumbed to intracranial challenge[135,136,183]. However, intracranial inoculation does not reflect the natural mpox transmission route. Since then, there have been several mouse studies to identify the ideal strain, route of exposure and doses to study pathogenesis, and vaccine and antiviral drug development.

Studies from initial decades of MPXV research used outbred white mice with limited genetic characterization. Osorio et al.[184] and Hutson et al.[168] were the first to compare the pathogenicity of two MPXV clades in two commercial inbred mouse strains, namely BALB/c and C57BL/6. Osorio et al. used the intraperitoneal route for viral challenge and also included SCID BALB/c and SCID C57BL/6 mice in their study, while Hutson et al. used subcutaneous injection in the footpad (f.p.) or the intranasal route to administer the virus. Both studies infected mice with 10^5 PFU of virus. F.p. exposure of both strains with the two different clades resulted in edema but no significant body weight loss, and all mice survived infection. With i.n. infection of clade I, however, both BALB/c and C57BL/6 mice lost body weight between days 0–9 post-infection (mean loss: 9.14% and 8.16%, respectively), without other overt signs of illness or lesions; again, all mice survived. With clade IIa via the i.n. route, no signs of morbidity were observed. Osorio et al. additionally used a recombinant MPXV expressing the luciferase gene to visualize disease progression *in vivo* via biophotonic imaging following i.p. exposure. In immunocompetent BALB/c and C57BL/6 mice, infection produced only mild clinical signs, including decreased activity, inappetence and rough coat, from which the mice recovered quickly. In contrast, SCID BALB/c and SCID C57BL/6 mice developed systemic clinical manifestations, and all mice died by day 9 (clade I) or day 11 (clade IIa) post-infection. *In vivo* imaging of these SCID mice further revealed that the virus was not confined to the peritoneal cavity and abdominal organs but disseminated to other sites, including the tail, feet, and nasal areas. Together, these two early studies concluded that MPXV infection in immunocompetent inbred BALB/c and C57BL/6 mice produced only mild clinical presentation and therefore would not suffice as a model for human mpox disease.

Identification of CAST/EiJ as a suitable mpox model

In 2010, Americo et al. repeated the experiments in BALB/c strain with different doses of MPXV and found that the LD_{50} of $>10^7$ PFU/mouse for clade I in this strain of mice was too high for practical study designs. Therefore, the authors made an attempt to identify a mouse strain suitable for use as a model for mpox. The search included testing 37 strains of mice for susceptibility to MPXV. Of the 37 strains, only three showed body weight loss above 20% at 2×10^4 PFU: CAST/EiJ, MOLF/EiJ, and PERA/EiJ with lethality of 100%, 75%, and 40% respectively. Because CAST/EiJ was more readily available and highly susceptible, this strain was further evaluated as a potential small animal model.

CAST/EiJ mice are a wild-derived inbred strain of *Mus musculus castaneus*, originally trapped at a grain storage facility in Thailand. *M. m. castaneus and M. m. domesticus*, the subspecies from which most classical laboratory strains are derived, diverged from a common ancestor approximately one million years ago. CAST/EiJ mice carry a markedly higher density of polymorphisms relative to classical inbred strains. CAST/EiJ is one of the eight founder strains of the Collaborative Cross panel [178,185,186].

Dose escalation/response studies in CAST/EiJ with intranasal infection of clade I revealed that this mouse strain was highly sensitive to MPXV. Mice started losing weight at doses as low as 10^2 PFU, with observed lethality of 60%, 100%, and 100% at 10^3, 10^4, and 10^5 PFU respectively. Following intraperitoneal inoculation of the same virus, disease severity was heightened; lethality was observed at doses as low as 10^1 PFU with an LD_{50} of 14 PFU, which was more than 1.5 $\log_{10}$ lower than the LD_{50} observed for i.n. infection (680 PFU). Virus was recovered at high titers from lungs, liver and spleen in both infection groups. However, no skin lesions or rashes were observed. Scarification on the shaved back or footpad inoculation of virus

resulted in no overt signs of disease such as hunched posture or ruffled fur, although footpad exposure resulted in leg swelling. Intranasal infection with clade IIa resulted in similar disease as clade I but required more than 1 $\log_{10}$ higher dose to produce similar lethality. Lethality observed were 17%, 50%, 100%, and 100% at 10^3, 10^4, 10^5 and 10^6 PFU respectively; unlike clade I, 10^2 PFU produced no overt signs of disease. These observations in CAST/EiJ mice are consistent with the evidence that clade I is more virulent than clade II in human cases and in other experimental animal models. This provides opportunities to study the pathogenic differences between clades at the genetic level using this mouse model.

Similarly, CAST/EiJ mice were fully protected against lethal MPXV challenge when immunized with DryVax, a licensed smallpox vaccine derived from live VACV, and when treated with cidofovir, an inhibitor of orthopoxvirus DNA replication. Upon evaluation of immune competence, CAST/EiJ mice exhibited CD8 and CD4 T-cell responses to VACV that were either comparable to or superior to those of BALB/c mice, along with higher VACV-specific IgG titers when immunized with DryVax. Together, it demonstrates that the CAST/EiJ mice are competent in their ability to mount acquired immune response and thus can be used as a viable model for immunological and antiviral studies[178].

Host immune factors underlying CAST/EiJ susceptibility to MPXV

The majority of our understanding of CAST/EiJ sensitivity to MPXV comes from the work contributed by Patricia L. Earl, Jeffrey L. Americo and Bernard Moss over more than a decade following their initial discovery of this mouse strain's high susceptibility to MPXV and the recognition of the opportunity to develop and characterize it as a fully functional mouse model. Initial investigations focused on identifying host factors underlying the sensitivity of

CAST/EiJ mice to MPXV, while the majority of other classical laboratory mouse strains remained resistant. The researchers systematically investigated host immune factors to compare and explore differences in immune responses to MPXV between susceptible CAST/EiJ and resistant classical inbred mouse strains. The first study reported that CD8 T cell and antibody levels against MPXV were similar in both BALB/c and CAST/EiJ mice, suggesting that CAST/EiJ mice were competent to mount an acquired immune response[187]. Subsequent investigation was then directed toward the innate immune response. In their next study after identification of CAST/EiJ as a susceptible strain to MPXV, the group reported that CAST/EiJ sensitivity was associated with deficient interferon-gamma (IFN-γ) response following MPXV infection. When lung cytokine levels were compared between resistant BALB/c and susceptible CAST/EiJ mice after lethal dose infection with MPXV, which produced similar viral kinetics in lungs, IFN-γ levels were significantly lower in CAST/EiJ. This deficiency resulted in widespread viral dissemination to vital organs and 100% mortality in CAST/EiJ, whereas BALB/c exhibited minimal viral dissemination with virus largely restricted to lungs and full recovery from infection. In contrast, treatment of CAST/EiJ mice with exogenous IFN-γ before and during MPXV infection resulted in several-fold lower viral titers in lungs and complete protection against disease. Given the established role of IFN-γ in controlling poxvirus infections, the deficient IFN-γ production and poor induction of CCL5 in CAST/EiJ lungs suggests that the innate immune system, particularly interferon signaling, represents a major factor whose deficiency contributes to the heightened sensitivity of CAST/EiJ to MPXV[187–192].

Expansion of susceptibility testing in CAST/EiJ mice to other orthopoxviruses by the group found that these mice are also highly susceptible to VACV and cowpox virus. VACV infection resulted in a disease course similar to that seen with MPXV, and CAST/EiJ mice were

more susceptible than BALB/c mice following intranasal infection. Because VACV is a BSL-2 agent, it does not require the stringent biosafety and containment measures needed for MPXV. As a result, researchers used VACV as a surrogate for MPXV to evaluate immune responses in CAST/EiJ mice[193,194].

Upon intraperitoneal infection with VACV, CAST/EiJ and BALB/c mice showed a stark contrast in disease outcome: CAST/EiJ mice were highly susceptible, exhibiting weight loss and lethality even at low doses while BALB/c mice showed only mild disease with 100% survival and no weight loss. This difference of high susceptibility in CAST/EiJ versus strong resistance in BALB/c was attributed to a relative deficiency in the immediate innate immune response of CAST/EiJ mice compared to BALB/c mice. They found that the IFN-γ and TNF-α responses following VACV infection or inoculation with poly(I:C), a double-stranded RNA mimic and Toll-like receptor 3 (TLR3) ligand, were higher and more rapid in BALB/c mice than in CAST/EiJ mice[194]. NK cells are key immune cells activated early during viral infection and are major early contributors of IFN-γ and TNF-α. Their role in limiting orthopoxvirus infection is well established [195–198]. CAST/EiJ mice have a relative deficiency in NK cells compared to other mouse strains. According to the Mouse Phenome Database (http://www.jax.org/phenome), among the 32 inbred mouse strains evaluated for peripheral blood cell phenotypes, CAST/EiJ ranked 29th in terms of NK cell population[199]. The authors also observed low baseline levels of NK cells, as well as CD4 and CD8 T cells, in the spleens of naïve CAST/EiJ mice compared to BALB/c mice, which could help explain the diminished IFN-γ response and greater susceptibility during early infection[194].

In a subsequent study, the authors further confirmed that NK cell deficiency in CAST/EiJ mice contributes to enhanced orthopoxvirus infection. They found that passive transfer of

purified NK cells *ex vivo* or expansion of NK cell numbers via IL-15 treatment *in vivo* resulted in suppressed viral replication and reduced lethality. The ability of CAST/EiJ mice to respond to IL-15 stimulation by producing and sustaining NK cells suggests that their low NK cell numbers are not due to an inability of IL-15 to interact with receptors on progenitor cells but rather to deficient NK cell homeostasis in the periphery[200].

Application of CAST/EiJ mice in mpox research

CAST/EiJ mice have thus been extensively used in mpox research due to their high susceptibility to MPXV. Experimental studies involving several exposure routes, including intranasal, intraperitoneal, and intradermal, and different clades (Ia, Ib, IIa, and IIb) have been conducted in this mouse strain to characterize disease progression, viral shedding and immune responses[33,178,179,187,201] (as described in a later subchapter). However, infection via mucosal routes, particularly rectal and genital, in this strain of mice is lacking, despite these routes becoming more epidemiologically relevant given the central role of intimate sexual contact in modern mpox transmission. This gap presents an opportunity to establish CAST/EiJ mice as a model for mucosal infection and transmission of MPXV.

Other animal models

Attempts to model MPXV infection have also been made in a range of other animal species. Guinea pigs (*Cavia porcellus*) did not develop disease when exposed to MPXV via several routes, including intracardial[183], intravenous[11,133], subcutaneous[11,133], intraperitoneal[11,133], intradermal[133] and oral[183]. Within the family *Cricetidae*, hamsters (*Mesocricetus auratus*)[183] and deer mice (*Peromyscus maniculatus rufinus*)[202] were found to be refractory to MPXV exposure,

whereas the cotton rat (*Sigmodon hispidus)*[203] was susceptible, showing 100% and 50% mortality via the intravenous and intranasal routes, respectively, accompanied by clinical signs such as cough, rhinitis, conjunctivitis and cyanosis. The multimammate rat (*Mastomys natalensis*) was more recently evaluated as a potential mucosal infection model, aiming to characterize mucosal mpox disease and the sexual/intimate contact transmission observed during the recent outbreak[204]. Genital and rectal inoculation with MPXV clade IIb resulted in sustained viral replication and shedding at the respective mucosal sites. Susceptibility of rabbits (*Oryctolagus cuniculus*) to MPXV depends on both animal age and route of exposure: young animals are more susceptible than adults, and disease is most severe following intracerebral and intravenous inoculation[203]. Skin lesions were observed in rabbits when infected via several routes, including intravenous[133,135,183], scarification[183], intradermal[183,205] and oral exposure[183]. The African/Gambian pouched rat (*Cricetomys gambianus)* was also investigated as a potential mpox model, prompted by the linkage of this imported African species to prairie dog infections during the 2003 US outbreak. Scarification or intradermal exposure in this species resulted in 12–33% mortality, along with clinical signs including skin lesions[206,207]. The disease has been experimentally evaluated in domestic pigs (*Sus scrofa*), in which combined intravenous, intradermal , and intranasal exposure produced subclinical infection with sparse cutaneous lesions around the facial region, and the virus was transmitted to contact animals[208].

Detailed characterization of MPXV infection in susceptible animal species, including viral pathogenesis, immune responses, and transmission dynamics, is important, as it provides valuable information for controlling and preventing disease spread and for developing effective drugs and vaccines. Different models offer distinct advantages for characterizing different

aspects of mpox disease and no single model is likely to address all research questions, therefore an integrated approach combining multiple models is pivotal. Practical factors such as cost, availability, housing and breeding requirements and handling under biocontainment conditions must also be considered when selecting an appropriate model. Furthermore, given the diverse and wide host range of MPXV, investigating the disease in novel animal species remains equally important.

Orthopoxvirus disease in *Bovidae* and other livestock animals

Orthopoxviruses are a group of phylogenetically and antigenically related viruses with a wide range of animal reservoirs, spanning from small rodents to large livestock species. While some members are strictly host-restricted, such as variola virus in humans, the causative agent of smallpox, and ectromelia virus in mice, the majority of orthopoxviruses exhibit broad host range. Most members are zoonotic in nature and can infect humans following spillover from infected animals via direct contact, respiratory droplets, or consumption of bushmeat[1,209,210].

Among the 17 recognized orthopoxvirus species, only a few are known to cause disease in cattle and other livestock species. These diseases are typically exanthematous and pustular in nature[1]. There are no reports of natural mpox infection in livestock species to date.

Bovine vaccinia virus

Exanthematous disease in cattle, producing vesicular and pustular lesions, was first recognized in Brazil as early as the 1960s and was known to also infect humans. The disease was caused by a virus closely related to human VACV and termed bovine vaccinia (BV). BV incidence increased in the 1990s and the disease is endemic within Brazil[211–213]. In recent years, several BV outbreaks have been reported on dairy cattle farms in Brazil. Two distinct VACV lineages circulate in cattle, group 1 and group 2, with group 1 predominating[211,214,215]. Similar VACV-associated disease in cattle has also been reported in other South American countries, including Argentina[216], Colombia[217] and Uruguay[218].

In cattle, BV primarily affects the teats and udder, producing characteristic pox lesions. Clinically, red papules begin to appear as early as two days after viral introduction on the skin of the teats and udder, progressing through vesicles, umbilicated pustules, and ulcers before

forming scabs, which resolve within 15 to 30 days. Clinical signs include reduced milk production and increased susceptibility to secondary infections and mastitis. Suckling calves become infected via contact with the udder, developing lesions on the muzzle, lips and oral mucosa[211,219].

Bovine vaccinia is a zoonotic disease, with frequent outbreaks reported in humans in Brazil in recent years. Farmers and rural workers in direct contact with cattle represent the most affected population, with infection often acquired through contact with the teats and udders of lactating cattle. Human infection typically presents with pox lesions on the hands, fingers, arms, and sometimes the face, with clinical signs such as headache, anorexia, and lymphadenopathy[1,220]. The disease is self-limiting and generally resolves within two to three weeks, although severe cases requiring hospitalization have been reported[212,221–223].

Buffalopox virus

Buffalopox virus (BPXV), another member of the orthopoxvirus family and a close genetic variant of VACV, causes buffalo pox in buffaloes and cattle. Buffalopox was first described and isolated in India more than half a century ago, and it is now recognized as an emerging disease, with frequent sporadic outbreaks reported in domestic buffalo and cattle herds across India and other south Asian countries. BPXV is believed to have emerged and evolved from the Lister vaccine strain of VACV, the strain historically used to produce smallpox vaccine in buffaloes[224–226]. Zoonotic infection with BPXV in humans has also been frequently reported, with patients developing pox lesions on the hands and forearms, usually accompanied with general malaise and fever. In buffalo, BPXV typically produces localized self-limiting pox

exanthema with lesions on the udder, teats, muzzle and other areas of the head, and it has a negative impact on milk productivity[224,227].

Cowpox virus

Cowpox derives its name from the disease it originally caused in the udders of cattle, along with associated zoonotic infection on the hands of milkers. However, the disease is no longer common in cattle and is likely endemic in rodents across Eurasia, with spillover to felids, which likely act as amplifying host. The disease has been reported in a range of wild and zoo animals, more frequently in felids[228,229]. In rodent species, infection is typically subclinical under both field and experimental conditions, but severe and sometimes fatal systemic disease has been reported in felids[228,230]. The last reported case of cowpox outbreak in cattle occurred decades ago[229,231]. However, given that cattle remain susceptible to cowpox virus, the possibility of disease re-emergence in this species cannot be ruled out.

Akhmeta virus

In 2013, a novel orthopoxvirus named Akhmeta virus, phylogenetically distinct from all previously known orthopoxviruses, was identified in two cattle herders in Akhmeta, Georgia, who were initially suspected of cowpox virus infection. Investigation of cattle belonging to these herders found no evidence of active infection, as cattle samples were negative for viral DNA and live virus; however, serum samples tested positive for anti-OPXV IgG antibodies[232]. To date, active infection has not been detected in cattle, though surveillance in the region remains limited. The virus was later isolated from wild rodents in the region[233]. The susceptibility of cattle to this virus as well as the true prevalence of disease in the region remains unknown.

Camelpox virus

Camelpox is another emerging zoonotic orthopoxviral disease. Camelpox virus is more closely related to variola virus, the causative agent of smallpox, than to other orthopoxviruses. The virus was historically confined to camel herds in countries across Africa and Asia, until recently, when camelpox outbreaks were reported in camels in Syria, along with zoonotic spillover to humans in India[234,235], Sudan[236], Qatar[237] and Somalia[238]. Camel pox causes severe disease, with case fatality rates of up to 28% in adult camels and reaching 100% in young animals. The virus is host-specific and does not affect cattle[235,239].

Horsepox virus

Horsepox virus (HPXV) is another orthopoxvirus, causing pustular disease in horses. The virus is likely extinct; the last documented cases occurred in Mongolian horses in the 1980s, and in 2012, the disease was officially removed from the list of notifiable diseases by the World Organization for Animal Health (WOAH)[240,241]. In 2017, however, a group of researchers reconstructed infectious horsepox virus from chemically synthesized DNA fragments, with the aim of stimulating development of HPXV-based smallpox vaccines[242].

Monkeypox virus in swine

Swine represent another major livestock industry alongside cattle. Pigs are naturally susceptible to Swinepox virus, a member of a distinct genus, *Suipoxvirus*, within the family *Poxviridae*, which causes a sporadic disease of eruptive dermatitis in pigs. However, natural orthopoxvirus infection in *Suidae* has not yet been reported[243]. Mantlo et al. experimentally

infected domestic pigs with a 2022 MPXV clade IIb isolate to investigate the susceptibility and transmissibility of the virus in swine. Infected pigs developed lesions around the lips and nose, with MPXV DNA and antigen detected within these lesions. Viral DNA was also detected in nasal and oral swabs from infected animal. Sentinel animals introduced and co-housed with infected pigs two days post-challenge also developed skin lesions with detection of MPXV DNA in their swabs together with an anti-MPXV antibody response which confirmed transmission of infection to these sentinel animals[208].

Monkeypox virus in *Bovidae*

The *Bovidae* family, which includes cattle, was not previously considered susceptible to MPXV until its recent detection in duikers in Africa in 2022. MPXV was first detected in the meat of Weyns's duiker (*Cephalophus weynsi*) and common duiker (*Sylvicapra grimmia)* sold in bushmeat markets in the Democratic Republic of the Congo, as part of a surveillance study of wild meat sold in local bush markets. Further investigation of duiker tissue samples collected between 2017 and 2024 in Taï National Park, Côte d'Ivoire, revealed MPXV positivity in a Maxwell's duiker (*Philantomba maxwellii)* carcass. Detection of MPXV in duikers across both Central and West Africa suggests that these animals are susceptible to natural mpox infection[130].

MPXV Clade IIb pathogenesis in animal models

For several decades, mpox was confined to endemic regions, with the virus circulating in wildlife and causing sporadic zoonotic spillover into human communities in rainforest areas. Over the past decade, however, there has been a rise in human mpox cases within the African region, as well as travel-associated exportation of the disease to other continents. In 2022, a large-scale global outbreak of mpox occurred, caused by a newly emerged subclade of the virus, clade IIb, predominated by lineage B.1[44]. In contrast to endemic mpox, the 2022 outbreak had unique epidemiological features. Men having sex with men (MSM) were the most affected demographic, with sexual contact serving as the major transmission route driving efficient human-to-human spread. Similarly, the disease was generally mild, and clinical presentation was less severe and, in some cases, atypical[57]. Mutations in the newly emerged clade have been suggested to contribute to reduced virulence and disease attenuation, as well as enhanced human-to-human transmissibility. The mutation rate observed in clade IIb since 2017 has been far greater than expected for the orthopoxviruses. It is believed that APOBEC3-driven mutagenesis has facilitated the adaptation of this novel virus strain to humans, driving efficient human-to-human transmission[20,34,44,244]. Much remains to be explored regarding this outbreak and the genetically novel, evolving virus behind it.

Animal models are an important tool that allow researchers to better understand the pathophysiology of infectious diseases. Several experimental studies using different animal models (which are discussed below) have sought to characterize the novel 2022 outbreak strain of MPXV, aiming to better understand the underlying pathogenesis of disease, immune responses and clinical features associated with this newly emerged virus.

Nonhuman primate models

Nonhuman primates (NHPs) are known to best recapitulate mpox disease in terms of disease progression and clinical features compared to other animal models. Li et al. infected cynomolgus macaques via intraocular, intraoral, intrarectal and subcutaneous routes simultaneously with a 2022 outbreak isolate of MPXV clade IIb. Animals began showing clinical signs and lesions starting on day 3 post-infection (dpi), which worsened around days 7–9, accompanied by systemic illness such as anorexia, listlessness and papulovesicular lesions, resolving by day 14. The disease course was self-limiting and less severe overall than previously observed in NHPs infected with older clades (clade I and IIa). Clinical features closely resembled those observed during the 2022 human outbreak. Fewer lesions were reported. Skin rashes, anogenital rashes and mucosal lesions were consistent across all animals and spread systemically. Substantial viral shedding was observed in throat swabs, nasal swabs, rectal swabs, blister fluid and ocular swabs, with viral DNA detected in major internal organs and lymphatic systems[162].

Another study in NHPs provided more detailed insight into viral replication in rectal mucosa following rectal challenge, supporting the hypothesis that active localized viral replication in rectal and genital mucosa with sustained viral shedding contributes to human-to-human transmission following intimate sexual contact. Herate, et al. infected cynomolgus macaques via the rectal route with a 2022 outbreak isolate of MPXV[245]. Clinical signs were consistent with those observed during the human outbreak, including localized proctitis, rectal exudate and skin lesions in the genital and oral regions, all of which resolved within a short period. High and prolonged viral shedding was observed in rectal fluid and also in seminal plasma for up to three weeks, indicating viral dissemination to and replication within the genital

tract. Tissues of the digestive tract, including the rectum, colon and duodenum, along with associated lymph nodes, tested positive for viral DNA and infectious viral particles. Pustular and ulcerative lesions were present in the colon, and colorectal lesions were characterized by marked inflammatory infiltration of neutrophils and lymphocytes. *In situ* hybridization and immunohistochemistry detected the presence of viral RNA within these lesions, supporting active viral replication at this mucosal site[245].

Rodent models: systemic and aerosol routes of infection

Americo et al. identified CAST/EiJ strain of mice as a suitable small animal model for mpox after testing of 37 commercially available mouse strains[178]. Since then, most experimental mpox research has been conducted in CAST/EiJ mice. This model has been discussed earlier. The same group was the first to inoculate CAST/EiJ mice with a clade IIb isolate from the 2022 outbreak. In that study, three different mpox clades were compared for virulence and disease severity. Intranasal (i.n.) exposure with clade I produced disease with weight loss at doses as low as 10^2 PFU and lethality beginning at 10^3 PFU, with no survival at 10^4 PFU. Intraperitoneal (i.p.) infection with the same clade was more severe, with lethality observed at doses as low as 1 PFU and no survival at 100 PFU. Similarly, i.n. infection with clade IIa resulted in 80% mortality at 10^3 PFU and 100% at 10^4 PFU, while i.p. exposure with the same clade produced lethality starting at 10^3 PFU. In contrast, clade IIb was markedly attenuated compared to the older clades. No weight loss or clinical signs of disease were observed at doses up to 10^5 PFU via either the i.n. or i.p. route. In the clade IIb i.n. exposure group, only a few mice had detectable virus in lungs; no virus was detected in other organs, whereas other clades had detectable virus in lungs, liver, and spleen. Similarly, with i.p. infection, virus was detected at very low levels in lungs,

liver, and spleen with clade IIb compared to other clades, which had 100-fold higher titers in those organs[179].

Meyer zu Natrup et al. also repeated the i.n. inoculation experiment in CAST/EiJ mice with two different clades (IIa and IIb) of virus at 2×10^5 PFU per mouse and found similar results. Clade IIa infection resulted in clinical disease manifestation with body weight loss and 100% lethality by day 6 dpi with high viral loads in lungs, liver and spleen. In contrast, clade IIb infection resulted in complete survival with no body weight loss or clinical signs. Upon evaluation of lung cytokines, higher levels of IFN-α1, IFN-α2 and IFN-β were detected in clade IIb-infected animals[33].

Rodent models: skin scarification route of infection

Rash is the most commonly reported cutaneous symptom of mpox and plays an important role in disease transmission. The skin scarification or intradermal route closely mimics skin-to-skin transmission and produces hallmark cutaneous lesions. Meyer zu Natrup et al. inoculated CAST/EiJ mice with two clades of virus (clade IIa and IIb) at two doses (2×10^4 and 2×10^5 PFU) using skin scarification on the tail. Comparable body weight loss was observed in both clades when compared at each dose; though weight loss was more severe and biphasic at 2×10^5 PFU for both clades. Lethality, however, differed; at the lower dose, only clade IIa produced lethality; at the higher dose, both clades produced lethality. All mice exhibited clinical signs including ruffled fur and respiratory symptoms. Skin lesions were more pronounced and spread from the tail, the site of exposure, to other body areas at the higher dose. At low doses, infectious virus was not detected in lungs and liver for clade IIb but was detected for clade IIa at death or study endpoint (12 dpi). At higher doses, infectious virus was detected in internal organs for both

clades; however, titers were substantially higher for clade IIa. This suggests that virus can disseminate from the inoculation site at the tail to internal organs. At two timepoints when oropharyngeal swabs were collected (4 dpi and death or study endpoint), infectious virus was not detected at day 4 but was detected at endpoint with high titers for clade IIa compared to clade IIb. In contrast, viral titers were similar in skin swabs for both clade IIa and IIb-infected mice[33].

In a repeated experiment infecting CAST/EiJ mice with both clades at high dose (2×10^5 PFU) with an earlier endpoint (8 dpi), they observed similar morbidity and clinical features. Substantial titers of infectious virus were detected in organs, oropharyngeal swabs and skin swabs at the study endpoint. The study also analyzed immune response against the virus and provided important insights into the attenuation of clade IIb in CAST/EiJ mice. Mice infected with clade IIb mounted a stronger early neutralizing response compared to those infected with clade IIa. Similarly, levels of various cytokines including IL-6, IFN-α1, IFN-α2 and IFN-β were higher in clade IIb-infected mice compared to clade IIa-infected mice, and TNF-α was activated at substantially higher levels in clade IIb. The robust and early activation of the MPXV-specific immune response facilitated early and efficient viral clearance in clade IIb-infected mice, thereby resulting in the attenuated clinical outcome[33].

Rodent models: mucosal routes of infection

Intimate sexual contact was the predominant route of MPXV transmission during the 2022 clade IIb outbreak, particularly within MSM communities. The routes discussed above (i.n., i.p., i.d.) do not accurately mimic this mode of human transmission and fail to fully recapitulate mpox disease and clinical features involving infection via sexual contact. Rectal and vaginal administration of the virus are therefore more epidemiologically relevant and could

provide important information about the clinical features, shedding patterns and transmission dynamics directly relevant to the routes driving the recent outbreak.

Port et al., shortly after the 2022 outbreak, modeled the sexual transmission routes of MPXV clade IIb in multimammate rats (*Mastomys natalensis*). Rats were inoculated with a 2022 outbreak isolate of MPXV via intraperitoneal, rectal, vaginal, aerosol or transdermal routes. None of the routes produced overt clinical signs, and only the transdermal route produced transient, localized skin lesions. Redness and swelling were observed around the inoculation site in rats infected via the rectal and vaginal routes. Vaginal inoculation resulted in high levels of MPXV in the reproductive organs and gastrointestinal tract at the 8 dpi necropsy. Similarly, vaginal and uterine lesions were observed in the vaginally inoculated group, and rectal lesions were observed in the rectally inoculated group. These lesions exhibited typical pox-associated pathology, along with detectable MPXV antigen and RNA, suggesting active viral replication in the associated mucosal tissues. High levels of viral shedding were observed in oral and rectal swabs from rectally infected rats, as well as in oral and vaginal swabs from vaginally infected rats. In a subsequent experiment investigating direct-contact transmission among co-housed donor and sentinel animals following transdermal, rectal, and aerosol infection, the authors found that transmissibility in this strain of rat was low and inconsistent across all routes tested[204].

Chapter 2 - Experimental infection of cattle with Monkeypox virus

Introduction

Mpox is an emerging and re-emerging zoonotic disease caused by monkeypox virus (MPXV), a member of the genus *Orthopox*virus within the family *Poxviridae*. The virus primarily replicates in a wide range of rodents and small mammals in endemic regions of Africa. To date, mpox has not been reported in livestock species, including cattle[15]. However, other orthopoxviruses, such as bovine vaccinia virus, cowpox virus, and buffalopox virus, are known to naturally infect cattle, producing exanthematous and pustular disease along with reduced milk production[1,211]. Duikers, which belong to the same family as cattle (*Bovidae*), were recently found to harbor MPXV in the wild, following detection of the virus in duiker meat sold in bushmeat markets in the Democratic Republic of the Congo (DRC) as well as in duiker carcasses collected in a national park in Côte d'Ivoire[130]. These findings of natural orthopoxvirus infection in cattle alongside MPXV detection in a closely related *Bovidae* species, raise the question of whether cattle themselves are susceptible to monkeypox virus infection.

Understanding bovine susceptibility to mpox is particularly relevant given the global emergence of mpox in 2022 and the resurgence of outbreaks in endemic regions, especially the DRC since 2023. As transmission has shifted from sporadic cases and small clusters to larger outbreaks and multi-country epidemics, the risk of virus introduction into animal populations outside its endemic regions has increased[57,246]. Domestic animals and livestock may be particularly vulnerable, given their close and frequent contact with humans. Evidence of human-to-dog transmission of MPXV was recently reported in France[46], and experimentally infected pigs have also been shown to be susceptible to the virus[208]. Spillover events could enable viral adaptation and the establishment of new animal reservoirs, as seen with the spillover of SARS-

CoV-2 into white-tailed deer, efficient deer-to-deer transmission and subsequent spillback events to humans[247,248]. Alternatively, infection of an unusual livestock host could result in significant disease and economic consequences, as demonstrated by the recent emergence of H5N1 in dairy cattle[249].

To evaluate susceptibility and transmission, calves were exposed with a MPXV clade IIb isolate from 2022 outbreak inoculated simultaneously via the intranasal, intradermal, and intravenous routes, followed by introduction of the sentinel animals two days post-inoculation of the calves.

Methods

Cells and virus

Vero E6 and MA104 cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; Corning) supplemented with either 5% or 10% fetal bovine serum (FBS; R&D Systems) and 1% antibiotic-antimycotic solution (Gibco). MA104 cells were used for viral propagation while Vero E6 cells were used for virological assays.

Monkeypox virus (MPXV) used in this study was obtained from BEI Resources (Catalog No. NR-58622), deposited by the Centers for Disease Control and Prevention (CDC). The virus belongs to clade IIb, lineage B.1 (strain hMPXV/USA/MA001/2022) and was originally isolated from a human clinical case in Massachusetts, USA, during the 2022 mpox outbreak. The complete genomic sequence of this strain is available through GenBank (Accession No. ON563414.3) and GISAID (EPI_ISL_13052289).

Vaccinia virus strain vTF7-3 [Wr] was obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH (ARP-356), contributed by Dr. Tom Fuerst and Dr. Bernard Moss.

Experimental group and virus inoculation

Eleven castrated male calves, 4.5 months of age and with a mean body weight of 146 kg, were acclimatized for three days in a BSL-3Ag facility before viral challenge, during which baseline temperatures and clinical samples were collected. Two animals, randomly assigned as baseline animals, were humanely euthanized prior to the start of the study. Six animals, randomly assigned as principal calves, were infected with MPXV clade IIb via simultaneous intranasal, intradermal, and intravenous inoculation. The remaining three animals were introduced to co-mingle with the principal calves at 2 days post inoculation (dpi) and served as sentinels to assess animal-to-animal transmission of the virus.

Principal calves received a total dose of administered 7.5×10^7 $TCID_{50}$ concurrently by three routes: intradermal injection into the skin folds at the base of the tail, intravenous injection into the left jugular vein, and intranasal instillation into both nostrils (1mL of $2.5*10^7$ $TCID_{50}$/mL per route).

Clinical observations

All animals were observed daily for development of clinical signs, assessed on the basis of four parameters: activity, appetite, respiratory distress and development of skin lesions. Rectal temperatures were recorded daily. Sentinel animals were observed and sampled first to minimize the risk of cross-contamination. A predefined clinical scoring system, based on an evaluation of

four parameters; activity, appetite, respiratory distress and lesion severity, was used to assess clinical disease progression.

Sample collection

Blood and swab samples (oral, nasal and rectal) were collected at 0, 1, 3, 5, 7, 10, 14, 17, and 20/21 dpi. Serum samples were collected at -1, 7, 14, and 20/21 dpi. Nasal swab sampling was performed using microbrush nasopharyngeal swabs (Young Innovations, #292496), which were gently inserted into both nares and rotated to ensure adequate sample collection. Oral swabs were collected using polyester-tipped swabs (ThermoFisher, #22363170) by swabbing the buccal mucosa. Polyester tipped swabs were also used to collect rectal swabs, where were obtained by inserting them into the rectal opening. Swabs were then placed into 1 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% antibiotic–antimycotic solution. Blood samples were collected from the jugular vein into EDTA tubes and/or serum separator vacutainer tubes. All swabs and blood samples were processed in the laboratory and stored at −80 °C until further analysis. Serum was separated according to the manufacturer's instructions. Briefly, samples were centrifuged at 1,500 × g for 10 min with full brake, and the serum was aliquoted and stored at -80 °C.

Two of the six principal animals were euthanized at 7 dpi, two at 14 dpi and the remaining at 20 dpi; sentinel animals were euthanized at 21 dpi. Necropsies were performed immediately following euthanasia and tissue samples were collected. Representative sections of each organ or tissue were placed into 5 mL tubes and stored at −80 °C for virological analyses.

All samples collected from animals were handled and analyzed in biosafety level 3 (BSL-3) laboratory facilities. Samples inactivated according to an approved viral inactivation protocol

were released to BSL-2 laboratories for downstream analysis following review and approval by the Institutional Biosafety Committee.

Nucleic acid extraction and qPCR

For clinical swabs, 200 µl of the specimen was added to ATL lysis buffer (Qiagen) at a 1:1 ratio. The mixture was briefly vortexed and incubated for 15 minutes. Tissue specimens were thawed, minced and added to ATL lysis buffer supplemented with 40µl of Proteinase K, with tissue weight adjusted to a concentration not exceeding 20% of the total buffer volume. The samples were then homogenized using a TissueLyser (4 pulses of 30 seconds each). The tissue homogenates were then subjected to heat treatment at 56°C for 40 minutes at 550 RPM. Both procedures were performed in accordance with the institutionally approved protocol for inactivation of infectious samples and following biosafety approval for release, inactivated samples were transferred from the BSL-3 to the BSL-2 laboratory for downstream processing.

Total DNA extraction was performed using the GeneReach Total Nucleic Acid Extraction Kit on the Taco Mini Prime Purification System (GeneReach, Taichung City, Taiwan) or BioSprint 96 workstation (Qiagen, Hilden, Germany). Briefly, for swab lysates, 200 µl of the sample was added to 500 µl of lysis buffer and 50 µl of magnetic beads. For tissue homogenates, a volume of 100–150 µl of the lysate was supplemented with 100 µl of PBS used for extraction protocol. All samples were eluted in a final volume of 100 µl.

To quantify viral load, a generic MPXV real-time PCR assay (G2R-G) was employed. This assay, developed by Li et al.[250], targets a conserved sequence within OPG002(G2R), which encodes the viral tumor necrosis factor receptor homologue CrmB and is located in the terminal inverted repeat (ITR) region of the viral genome. The oligonucleotide sequences were as follows:

forward primer, 5′-GGAAAATGTAAAGACAACGAATACAG; reverse primer, 5′-GCTATCACATAATCTGGAAGCGTA; and probe, 5′FAM-AAGCCGTAATCTATGTTGTCTATCGTGTCC-3′BHQ1.

qPCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using PerfeCTa® FastMix® II (Quantabio, Beverly, MA, USA). All qPCR reactions were set up in a final volume of 20 µL, containing 5 µL of DNA template, 0.4 µM of each primer and 0.2 µM of FAM-labeled probe (Integrated DNA Technologies, Coralville, IA, USA). Each sample was run in duplicate. Thermocycling conditions for the MPXV G2R_G assay consisted of an initial denaturation at 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 10 seconds and 60 °C for 1 minute. Each run included negative and positive controls consisting of molecular-grade water and a known MPXV-positive amplification control respectively. Cycle threshold (Ct) cutoffs were selected based on the analytical performance of the assay and CDC recommendations, with a Ct of 38 used for swab samples and a Ct of 40 for tissue samples.

Virus isolation

Virus isolation was performed on PCR-positive samples, which included a few nasal and oral swabs. Samples were thawed and vortexed, and 400 µL of each swab sample was diluted 1:1 in DMEM and passed through a 0.45 µm syringe filter. A total of 500 µL of the recovered filtrate was then inoculated in duplicate to individual wells of 6-well plates seeded with Vero-E6 cells, followed by gentle rocking to ensure even distribution of the inoculum. After a 2-hour incubation at 37 °C, 1,500 µL of DMEM supplemented with 2% FBS and 1% antibiotic–antimycotic

solution was added to each well, and cultures were incubated at 37 °C in 5% CO_2 and monitored daily from 2 days post inoculation for cytopathic effect (CPE).

After 5 days of incubation, the culture medium was removed and the cells were fixed with 80% acetone for 12 minutes. Next, 1 mL of rabbit anti-vaccinia virus (VACV) polyclonal antibody (PA1-7258, Invitrogen, Waltham, MA, USA) diluted in 1% horse serum albumin (HSA) in PBS was added and incubated for 1 hour at room temperature. Plates were washed twice with PBS-T (0.05% Tween-20) and once with PBS, after which 1 mL of Alexa Fluor 488 goat anti-rabbit IgG (1:1000 in 1% HSA in PBS; Invitrogen) was added and incubated for 1 hour at room temperature. Plaques were then visualized using an EVOS fluorescence microscope.

ELISA

Indirect ELISA using purified recombinant proteins A29 (Sino Biological Inc., Beijing, China) was performed to detect MPXV-specific antibodies in bovine serum. 300 ng of antigen in a final volume of 50 µL in coating buffer (carbonate–bicarbonate buffer, Sigma-Aldrich) was added to 96-well immunoassay plates (Maxisorp, Thermo Fisher) and incubated overnight at 4 °C. Plates were then washed three times with PBS. Wells were blocked with 100 µL of 5% non-fat dry milk in PBS for 1 hour, after which the blocking solution was removed and 50 µL of diluted serum samples was added. Samples were serially diluted twofold, starting at a 1:10 dilution. Following 1 hour of incubation at 37 °C, plates were washed twice with PBS-T and once with PBS, and 50 µL of goat anti-bovine IgG (H+L) HRP-conjugated secondary antibody (1:5000 in 5% non-fat dry milk; Thermo Fisher, #A18751) was added and incubated for 1 hour at room temperature. Plates were washed three times as described above, then 50 µL of TMB substrate solution (Thermo Fisher) was added and incubated for 15 minutes. The reaction was

stopped by adding 50 µL of stop solution (Thermo Fisher), and absorbance was measured at 450 nm using an ELISA plate reader. Samples were considered positive when the OD value exceeded the mean OD of naive serum plus three standard deviations.

Results

Calves remain subclinical following MPXV inoculation

Eleven male calves were grouped as follows: two baseline animals, six principally infected animals, and three sentinels. Baseline animals were humanely euthanized prior to the start of the study. The six principally infected calves were exposed simultaneously via intranasal, intradermal and intravenous routes with an MPXV clade IIb isolate obtained from human patient during the 2022 outbreak. Sentinel animals were co-housed with the principally infected animals two days post-infection to evaluate transmission. All animals were observed daily for clinical signs and lesions, and rectal temperature was monitored daily throughout the 21-day study period. Clinical samples were collected at regular intervals. Two principally infected animals were humanely euthanized at each of 7, 14, and 20 days post-inoculation (dpi), while sentinel animals were humanely euthanized on 21 dpi (Figure 2.1).

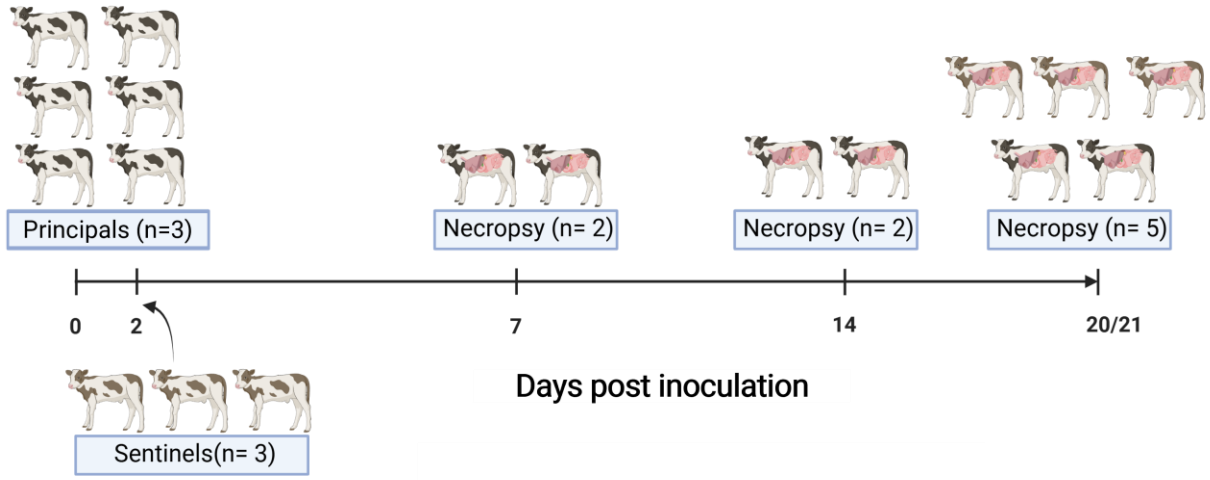


Figure 2.1 Experimental design for MPXV clade IIb inoculation of calves. Six male calves (principals) were exposed to MPXV clade IIb (hMPXV/USA/MA001/2022) at a total dose of 7.5×10^7 $TCID_{50}$, delivered simultaneously by three routes (1 mL of 2.5×10^7 $TCID_{50}$/mL per route): intradermal injection into the skin folds at the base of the tail, intravenous injection into the left jugular vein, and intranasal instillation into both nostrils. At 2 days post-inoculation (dpi), three uninoculated sentinel calves were co-housed with the principals (curved arrow). Whole blood and oral, nasal, and rectal swabs were collected at 0, 1, 3, 5, 7, 10, 14, 17, and 21 dpi. All animals were monitored daily for clinical signs including: appetite, activity, breathing and lesions, and rectal temperatures over the 21-day study. Two principals were humanely euthanized at each of 7, 14, and 20 dpi, and the three sentinels at 21 dpi. Created with BioRender.com.

All principally infected and sentinel animals exhibited normal activity, appetite, and respiration throughout the study period. Rectal temperatures in all animals remained within the physiological range, with no fever observed in any animal (Figure 2.2). However, most animals

developed skin lesions resembling ringworm on various parts of the body, particularly the face. Dermatophytosis, most commonly caused by *Trichophyton verrucosum*, is a common condition of calves in North America including USA and produces crusting lesions with focal alopecia[251–254]. A definitive diagnosis was not pursued in this study, and potential interference of dermatophyte lesions with MPXV detection was not evaluated.

Animal #6130 exhibited a round, irritated scar under the tail on day 4 post-inoculation (dpi). Similarly, animal #6129 developed a lesion on the left nostril on day 5 and 6 dpi, and animal #6134 developed a similar lesion on day 14 dpi. Animal #6135 had pinpoint lesions on the lower lip near the muzzle, while animals #6126 and #6128 had lesions on the tail near the inoculation site. All lesions were transient, present for only a short period. Skin lesions were collected on suspicion of MPXV infection but tested negative for MPXV DNA (Supplementary Table 1). No other overt signs or lesions consistent with mpox were observed.

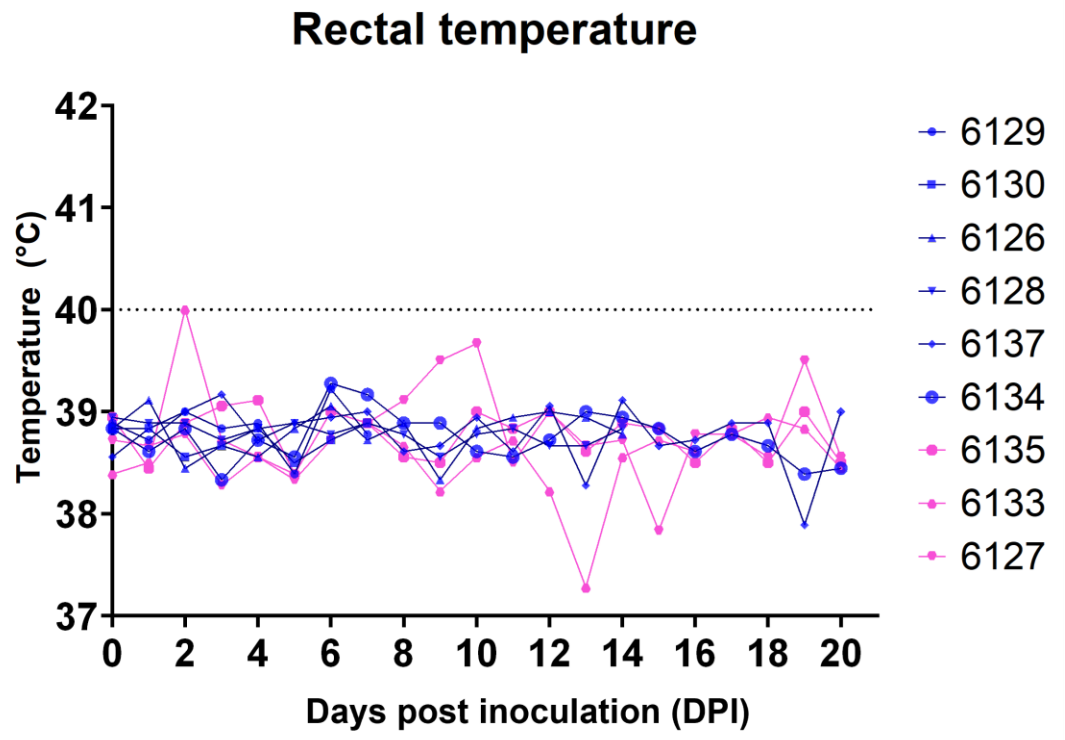


Figure 2.2 Rectal temperature of calves following MPXV clade IIb inoculation. Rectal temperatures were recorded daily from 0 to 21 days post inoculation (dpi). Each line represents an individual animal; principally infected calves are shown in blue and sentinel calves in pink. The dotted line indicates the febrile threshold (40 °C).

Limited viral detection in clinical samples and tissues

Oral, nasal, and rectal swabs, as well as blood, were collected at regular time intervals as described in methods section. Detection of viral DNA in these samples and tissues was performed using the G2R-G real-time PCR assay developed by the CDC[250]. Viral DNA was consistently detected in nasal swabs from two of the principally infected animals (#6129 and #6137) (Figure 2.3). In animal #6129, viral DNA was first detected at 1 dpi, peaked at 3 dpi, and remained detectable until the animal was humanely euthanized on 7 dpi. Similarly, in animal #6137, viral DNA was detected through 5 dpi but was not recovered thereafter until humanely euthanized on 20 dpi. Animal #6128 tested positive for MPXV DNA at 1 dpi only, which likely represents residual viral DNA at the site of inoculation. In oral swabs, detectable viral DNA shedding was observed in two animals (#6129 at 3 dpi, and #6130 at 1 and 3 dpi). In rectal swabs, only one animal (#6130 at 1 dpi) had viral DNA detected at a level above the assay cutoff. No animals had detectable viral DNA in blood at any timepoint.

qPCR positive samples were then subjected to viral isolation for the detection of any viable virus. MPXV-specific CPE were observed in nasal swabs of #6129 collected at 3 dpi and 5 dpi. The CPE was fixed and stained with a rabbit polyclonal antibody against VACV conjugated with AF-488. Both samples were positive for MPXV-specific staining, confirming the recovery of infectious virus.

In animal models of MPXV infection, the virus initially replicates at the site of inoculation before being carried to the draining lymph nodes and then spreading to major organs such as the liver and spleen, where it actively replicates before disseminating further to other organs[53,148,151,169,255]. Here, we collected a broad set of tissues, including major internal organs (liver, spleen, heart, lungs, kidney, gastrointestinal tract, brain, and others) and lymph nodes from multiple sites throughout the body. Skin samples, particularly from sites with visible lesions, were also collected at the time of necropsy. All collected tissues were subjected to the same PCR assay. No viral DNA was detected in any tissue collected from either the principally infected or sentinel animals (Supplementary Table 1).

A

Mean CT	Animal ID	1 DPI	3 DPI	5 DPI	7 DPI	10 DPI	14 DPI	17 DPI	21 DPI
Nasal Swab	6129	32.9	24.05	26.31	34.7				
	6130	ND	ND	ND	ND				
	6126	ND	ND	ND	ND	ND	ND		
	6128	35.78	ND	ND	ND	ND	ND		
	6137	35.37	26.68	28.86	ND	ND	ND	ND	ND
	6134	ND	ND	ND	ND	ND	ND	ND	ND
	6135		ND	ND	ND	ND	ND	ND	ND
	6133		ND	ND	ND	ND	ND	ND	ND
	6127		ND	ND	ND	ND	ND	ND	ND

B

Mean CT	Animal ID	1 DPI	3 DPI	5 DPI	7 DPI	10 DPI	14 DPI	17 DPI	21 DPI
Oral Swab	6129	ND	36.85	ND	ND				
	6130	37.57	32.59	ND	ND				
	6126	ND	ND	ND	ND	ND	ND		
	6128	ND	ND	ND	ND	ND	ND		
	6137	ND	ND	ND	ND	ND	ND	ND	ND
	6134	ND	ND	ND	ND	ND	ND	ND	ND
	6135		ND	ND	ND	ND	ND	ND	ND
	6133		ND	ND	ND	ND	ND	ND	ND
	6127		ND	ND	ND	ND	ND	ND	ND

C

Mean CT	Animal ID	1 DPI	3 DPI	5 DPI	7 DPI	10 DPI	14 DPI	17 DPI	21 DPI
Rectal Swab	6129	ND	ND	ND	ND				
	6130	37.47	ND	ND	ND				
	6126	ND	ND	ND	ND	ND	ND		
	6128	ND	ND	ND	ND	ND	ND		
	6137	ND	ND	ND	ND	ND	ND	ND	ND
	6134	ND	ND	ND	ND	ND	ND	ND	ND
	6135		ND	ND	ND	ND	ND	ND	ND
	6133		ND	ND	ND	ND	ND	ND	ND
	6127		ND	ND	ND	ND	ND	ND	ND

D

Mean CT	Animal ID	1 DPI	3 DPI	5 DPI	7 DPI	10 DPI	14 DPI	17 DPI	21 DPI
EDTA Blood	6129	ND	ND	ND	ND				
	6130	ND	ND	ND	ND				
	6126	ND	ND	ND	ND	ND	ND		
	6128	ND	ND	ND	ND	ND	ND		
	6137	ND	ND	ND	ND	ND	ND	ND	ND
	6134	ND	ND	ND	ND	ND	ND	ND	ND
	6135		ND	ND	ND	ND	ND	ND	ND
	6133		ND	ND	ND	ND	ND	ND	ND
	6127		ND	ND	ND	ND	ND	ND	ND

	Principally infected animals
	Sentinels
	Samples not collected
ND	Not detected

Figure 2.3 MPXV DNA detection by real-time PCR in swabs and whole blood. Mean cycle threshold (Ct) values for nasal **(A)**, oral **(B)**, and rectal **(C)** swabs and EDTA whole blood **(D)** collected from six principally infected calves (blue) and three sentinel calves (pink) at 1–21 days post inoculation (dpi). Each value is the mean of duplicate reactions of the MPXV G2R_G real-time PCR assay. Gray cells indicate samples not collected. dpi, days post inoculation; ND, not detected.

Minimal to no antibody response against MPXV in calves

Serum samples were collected on regular time-points and on the day of necropsy for serological examination. Indirect ELISA to detect serum antibody against recombinant MPXV protein A29 was used for antibody detection. A29 is a crucial protein for MPXV for viral attachment to host cell membrane and has been identified to elicit neutralizing antibodies. No A29 protein-specific IgG was detected in any sample (Figure 2.4).

To further assess orthopoxvirus-reactive antibodies in serum, samples were tested by indirect immunofluorescence assay (IFA) on VACV-infected Vero E6 cells, followed by staining with an anti-bovine IgG secondary antibody. MPXV and VACV belong to the same genus and share a highly conserved central coding region encoding homologous virion surface proteins that elicit cross-reactive antibodies, so VACV-infected cells serve as a surrogate antigen substrate for detecting anti-orthopoxvirus antibodies. Here, the result showed that no VACV-reactive IgG was detected in any of the sera tested (Figure 2.4).

A.

		-1 DPI	7 DPI	14 DPI	20/21 DPI
ELISA	6129	Neg.	Neg.		
	6130	Neg.	Neg.		
	6126	Neg.	Neg.	Neg.	
	6128	Neg.	Neg.	Neg.	
	6137	Neg.	Neg.	Neg.	Neg.
	6134	Neg.	Neg.	Neg.	Neg.
	6135	Neg.	Neg.	Neg.	Neg.
	6133	Neg.	Neg.	Neg.	Neg.
	6127	Neg.	Neg.	Neg.	Neg.

B.

		-1 DPI	7 DPI	14 DPI	20/21 DPI
IFA	6129	Neg.	Neg.		
	6130	Neg.	Neg.		
	6126	Neg.	Neg.	Neg.	
	6128	Neg.	Neg.	Neg.	
	6137	Neg.	Neg.	Neg.	Neg.
	6134	Neg.	Neg.	Neg.	Neg.
	6135	Neg.	Neg.	Neg.	Neg.
	6133	Neg.	Neg.	Neg.	Neg.
	6127	Neg.	Neg.	Neg.	Neg.

Figure 2.4 Serology of MPXV clade IIb-inoculated calves. Serum collected at −1, 7, 14, and 20/21 days post inoculation (dpi) from six principally infected calves (blue) and three sentinel calves (pink) was tested by **(A)** indirect ELISA against recombinant MPXV A29 protein and **(B)** indirect immunofluorescence assay (IFA) on VACV-infected Vero E6 cells, using an anti-bovine IgG secondary antibody. Samples were scored negative (Neg.) when the ELISA OD fell below the assay cutoff or no specific fluorescence was seen by IFA. Gray cells indicate samples not collected. dpi, days post inoculation; IFA, indirect immunofluorescence assay; VACV, vaccinia virus.

Discussion

In 2022, a global outbreak of mpox affected more than 100 countries, with over 100,000 confirmed human cases. This marked the first instance of worldwide dissemination of the virus in terms of both scale and geographical extent, raising concern that the virus could be introduced into animal populations outside its endemic regions and establish novel reservoirs[57,256]. Cattle are maintained in close contact with humans and are known hosts of related orthopoxviruses, including vaccinia virus, cowpox virus and buffalopox virus[1]. However, cattle susceptibility to MPXV has not been experimentally characterized. Here, we infected calves with a 2022 mpox outbreak isolate (MPXV clade IIb) via systemic, mucosal and cutaneous routes simultaneously. Infection remained subclinical, with no overt clinical signs of mpox. Cutaneous lesions were observed on the head/face and at inoculation sites but tested negative for MPXV DNA. No animal seroconverted, as sera were negative for both anti-MPXV and anti-orthopoxvirus antibodies.

Two of six principally infected animals (#6129 and #6137) showed consistent viral DNA detection in nasal swabs for at least three consecutive sampling points through 5 dpi, and infectious virus was recovered from nasal swabs of #6129 at 3 and 5 dpi. Viral DNA was likewise detected in oral swabs from #6129, together suggesting active local replication contributing to viral shedding in these animals. Upon further testing of oral and respiratory tract tissues, however, MPXV DNA was not detected in any tissue examined. Viral DNA detection in swabs peaked at 3 dpi and declined to low or undetectable levels by 5 dpi. Tissues were collected at scheduled necropsy timepoints no earlier than 7 dpi (#6129 at 7 dpi, #6137 at 20 dpi), by which time mucosal infection had likely resolved therefore the absence of detection in tissues should be interpreted with this timing in mind. Furthermore, virus was not recovered from any

internal organs or draining lymphatic tissues, suggesting transient and strictly localized viral replication with no evidence of systemic dissemination.

We observed apparent restriction of MPXV replication in cattle, a species susceptible to other viruses of the genus *Orthopoxvirus*. Orthopoxvirus genomes share a conserved central region across family members, while the flanking terminal regions are highly variable and contain genes that determine host specificity, virulence, and immune evasion[5,257]. Bovine vaccinia virus, buffalopox virus, and cowpox virus, all of which infect cattle, are phylogenetically more closely related to one another than to MPXV[1,258]. Additionally, relative to other orthopoxviruses, the MPXV genome has undergone progressive gene loss and truncation, and clade IIb lineage B.1 genome carry further deletions[21,259,260]. Divergence in host range, virulence and immunomodulatory determinants encoded by terminal regions may therefore contribute to the apparent restriction of MPXV replication in cattle.

These data suggest that cattle demonstrate minimal susceptibility to MPXV clade IIb, with no overt signs of disease and only limited viral shedding. Therefore, the ability of IIb to become endemic or a persistent nuisance to cattle in European breeds seems unlikely at this time. Nevertheless, these data demonstrate clinical, virological, and immunological parameters following inoculation that can be used for comparison for future studies or investigation of odd pox-like diseases on ranches and dairies. The virulence of MPXV clade IIb is markedly attenuated compared to other clades, producing only mild clinical signs even in susceptible animal models at high inoculation doses[33,162,179,245]. Thus, additional experiments are needed to determine if other clades are more virulent in cattle and other livestock species.

Chapter 3 - Mucosal infection of CAST/EiJ mice with Monkeypox virus

Introduction

In May, 2022, mpox was reported by countries outside the endemic regions of Africa. Over the following weeks, the disease, caused by the newly emerged lineage of subclade MPXV cladeIIb, spread rapidly across multiple continents with sharp surge in cases, which prompted the WHO to declare the 2022 mpox outbreak a Public Health Emergency of International Concern (PHEIC) on 23 July, 2022. The outbreak affected more than 100 countries across all six WHO regions with over 100,000 human mpox cases[18,44].

Several unique epidemiological and clinical features set the 2022 mpox outbreak apart from historical outbreaks, which were primarily zoonotic and confined to endemic regions of Africa. In contrast to endemic mpox, the 2022 outbreak was mild but marked by efficient human-to-human transmission. Men, particularly men who have sex with men (MSM), were the major demographic affected and intimate sexual contact within MSM communities was the predominant route of transmission. Prodromal signs and skin lesions were less frequently reported, and lesions were largely concentrated in the anogenital and oropharyngeal regions. Prolonged viral shedding was observed in rectal swabs, saliva and semen[57,261,262]. Studies exploring the underlying mechanisms of disease, the factors contributing to novel transmission route and atypical clinical presentation, and potential human adaptation of the virus remain limited.

CAST/EiJ mice have been established as a susceptible small animal model for mpox. Experimental studies using the 2022 outbreak strain of MPXV (clade IIb, lineage B.1) have evaluated infection through intraperitoneal, intranasal, and intradermal routes in this mouse

strain[33,179,201]. However, systemic and aerosol routes are becoming less epidemiologically relevant in current mpox transmission, given that intimate sexual contact has emerged as the major transmission route. Studies investigating mucosal routes of infection remain limited. Here, we infected CAST/EiJ mice with a 2022 human mpox isolate via four different routes (intraperitoneal, intranasal, rectal and genital) to investigate the susceptibility of CAST/EiJ mice to mucosal infection with MPXV clade IIb with the aim to establish this strain as a small animal model for mucosal transmission of mpox.

Methods

Cells and virus

Vero E6 cells (ATCC® CRL-1586™) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Corning) supplemented with either 5% or 10% fetal bovine serum (FBS; R&D Systems) and 1% antibiotic-antimycotic solution (Gibco). Cells were maintained at 37 °C, 5% CO_2.

Clade IIb, lineage B.1 monkeypox virus (MPXV) (strain hMPXV/USA/MA001/2022) was obtained from BEI Resources (Catalog No. NR-58622). Strain hMPXV/USA/MA001/2022 was originally isolated from a clinical human case in Massachusetts, USA, during the 2022 mpox outbreak. The complete genomic sequence is available through GenBank (ON563414.3) and GISAID (EPI_ISL_13052289). After receipt of the virus, it was passaged twice in Vero-E6 cells and harvested by three cycles of freeze-thaw, vortexing and sonication of both infected cells and supernatant. The viral titer was determined by plaque assay on Vero-E6 cells.

Experimental group and virus inoculation

Six-week-old CAST/EiJ mice were obtained from Jackson Laboratory. Mice were housed in groups of five males or five females in isolation cubicles under controlled temperature and light conditions and were provided ad libitum access to food and water. All animals were allowed to acclimatize for six days prior to the viral inoculation.

Mice were randomly assigned to one of five groups (five males and five females per group) based on the route of virus inoculation: intraperitoneal (systemic); intranasal, rectal, or genital (mucosal); or sham-infected (control). Mice in the inoculation groups were infected with 2×10^5 PFU of MPXV clade IIb (lineage B.1) via their respective routes, while the control group was sham-infected (Figure 3.1).

For the intraperitoneal inoculation, mice were administered 100 µl of virus inoculum using a 27G, 0.5 ml syringe (BD, REF 305620). Intranasal groups received a total virus inoculum volume of 40 µl, administered as 20 µl per nares using a micropipette. For rectal inoculation, 20 µl of the inoculum was instilled approximately 0.25-0.5 cm into the rectum using a 20 µl pipette tip; mice were then placed on their backs with their hindquarters elevated for a few seconds to ensure retention. Genital inoculation involved the administration of 10 µl of inoculum. In females, the inoculum was instilled into the vaginal cavity (intravaginal), and in males, it was instilled into the prepuce. Similar to the rectal group, these mice were held on their backs following administration. The control group was sham-infected intranasally with 40 µl of sterile phosphate-buffered saline (PBS).

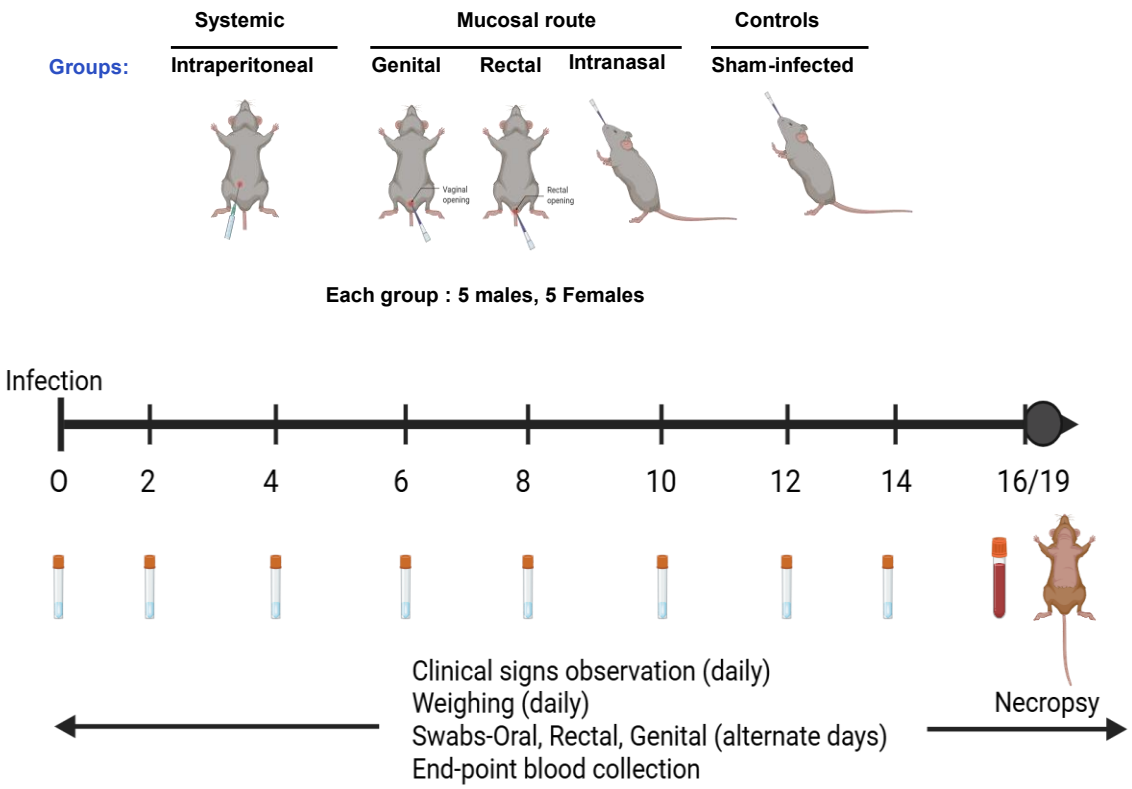


Figure 3.1 Experimental design for MPXV clade IIb inoculation of CAST/EiJ mice. Six-week-old CAST/EiJ mice were assigned to five groups (five males and five females per group) according to the route of virus inoculation: intraperitoneal (systemic); genital, rectal, or intranasal (mucosal); or sham-infected (control). Mice in the inoculation groups were infected with 2×10^5 PFU of MPXV clade IIb (strain hMPXV/USA/MA001/2022) via their respective routes. Oral, rectal, and genital swabs were collected on alternate days from 2 days post-inoculation (dpi), and all animals were monitored daily for clinical signs and body weight changes throughout the study. Mice in the intraperitoneal, intranasal, and control groups were humanely euthanized at 16 dpi, and those in the rectal and genital groups at 19 dpi, with end-point blood collection and necropsy performed at euthanasia. Created with BioRender.com.

Clinical observations

Following viral inoculation, clinical signs were evaluated daily prior to anesthesia for body weight records and sample collections. The clinical scoring system (Table 3.1) was predefined, and total scores were calculated from the cumulative assessment of four parameters: physical appearance, behavioral activity, respiratory effort and development of skin lesions. Each of these parameters was characterized by its presence or absence or by its clinical progression and was assigned a value on a scale of 0 (absent/normal) to a parameter specific maximum (most severe).

Table 3.1 Clinical scoring system for disease severity assessment in MPXV-inoculated mice. Clinical signs were evaluated daily and scored using a cumulative assessment of four parameters: appearance, activity, breathing, and skin lesions. Each parameter was independently scored based on the criteria listed, with scores ranging from 0 (normal/no signs) to parameter specific maximum of 3-5 (most severe). Individual parameter scores were combined to generate a cumulative clinical score for each animal, with higher scores reflecting greater disease severity.

Parameter	Monitoring criteria	Score
Appearance	Well groomed, smooth and silky coat	0
	Reduced grooming, slightly/mild rough coat	1
	Ruffled fur over the majority of back, rough coat	2
	Greasy fur, generalized piloerection, puffy appearance	3
Activity	Normal	0
	Slightly reduced activity	1
	Slow to move, slightly/mild hunched	2
	Hunched, some movement when stimulated/provoked	3
	Hunched, little or no movement even when stimulated/provoked	4
	Moribund	5

Breathing	Normal	0
	Moderately faster than normal	1
	Rapid breathing	2
	Labored breathing	3
	Labored breathing with gasps	4
Lesions	No lesions	0
	Small area of broken skin (<0.5cm)	1
	Area of broken skin between 0.5cm and 1 cm	2
	Broken skin, bleeding, scabbing, or pustules	3
	Broken skin >1 cm with multiple pustules and scabbing	4
	Severe scabbing or bleeding with pustules	5

Sample collection

Mice were anesthetized with isoflurane to effect prior to handling. Oropharyngeal, rectal and genital swabs were collected from each mouse every other day post-inoculation, beginning at 2 days post inoculation (dpi) and continuing through 14 dpi. Flocked nylon swabs (FLOQSwabs®, Copan Diagnostics) were used for sample collection. For oropharyngeal sampling, swabs were inserted into the oropharyngeal cavity ensuring contact with the oropharyngeal mucosa and then placed into 550 µl of Dulbecco's Modified Eagle Medium (DMEM; Corning) supplemented with 1% antibiotic–antimycotic solution (Gibco). Similarly, for rectal sampling, swabs were inserted into the rectal opening/anal orifice, gently rotated, and then transferred into the collection medium. For genital swab sampling, swabs were gently surface circumscribed across the urogenital opening to collect secretions. All samples were stored at −80 °C until analysis.

Mice in the intraperitoneal, intranasal and control groups were humanely euthanized at 16 dpi, while mice in the rectal and genital groups were euthanized at 19 dpi. Prior to euthanasia, blood was collected via cardiac puncture under deep isoflurane anesthesia. Necropsies were performed on the day of euthanasia to harvest the following tissues: liver, spleen, lungs, kidneys, colon, rectum and reproductive organs (testes and accessory reproductive tissues in males; ovaries and reproductive tract in females). Tissues were stored at -80°C for virological quantification.

All samples collected from animals were handled and analyzed in biosafety level 3 (BSL-3) laboratory facilities. Samples inactivated according to an approved viral inactivation protocol were released to BSL-2 laboratories for downstream analysis following review and approval by the Institutional Biosafety Committee.

Nucleic acid extraction and qPCR

For clinical swabs, 200 µl of the specimen was added to ATL lysis buffer (Qiagen) at a 1:1 ratio. The mixture was briefly vortexed and incubated for 15 minutes. Tissue specimens were thawed, minced and added to ATL lysis buffer supplemented with 40µl of Proteinase K, with tissue mass adjusted to a concentration not exceeding 20% of the total buffer volume. The samples were then homogenized using a TissueLyser (4 pulses of 30 seconds each). The tissue homogenates were then subjected to heat treatment at 56°C for 40 minutes at 550 RPM. Both procedures were performed in accordance with the institutionally approved protocol for inactivation of infectious samples and following biosafety approval for release, inactivated samples were transferred from the BSL-3 to the BSL-2 laboratory for downstream processing.

Total DNA extraction was performed using the GeneReach Total Nucleic Acid Extraction Kit on the Taco Mini Prime Purification System (GeneReach, Taichung City, Taiwan) or BioSprint 96 workstation (Qiagen, Hilden, Germany). Briefly, for swab lysates, 200 µl of the sample was added to 500 µl of lysis buffer and 50 µl of magnetic beads. For tissue homogenates, a volume of 100–150 µl of the lysate was supplemented with 100 µl of PBS used for extraction protocol. All samples were eluted in a final volume of 100 µl.

To quantify viral load, a generic MPXV real-time PCR assay (G2R_G) was employed. This assay, developed by Li et al.[250], targets a conserved sequence within OPG002(G2R), which encodes the viral tumor necrosis factor receptor homologue CrmB and is located in the terminal inverted repeat (ITR) region of the viral genome. The oligonucleotide sequences were as follows: forward primer, 5′-GGAAAATGTAAAGACAACGAATACAG; reverse primer, 5′-GCTATCACATAATCTGGAAGCGTA; and probe, 5′FAM-AAGCCGTAATCTATGTTGTCTATCGTGTCC-3′BHQ1.

qPCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using PerfeCTa® FastMix® II (Quantabio, Beverly, MA, USA). All qPCR reactions were set up in a final volume of 20 µL, containing 5 µL of DNA template, 0.4 µM of each primer and 0.2 µM of FAM-labeled probe (Integrated DNA Technologies, Coralville, IA, USA). Each sample was run in duplicate. Thermocycling conditions for the MPXV G2R_G assay consisted of an initial denaturation at 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 10 seconds and 60 °C for 1 minute. Each run included negative and positive controls consisting of molecular-grade water and a known MPXV-positive amplification control respectively. Cycle threshold (Ct) cutoffs were selected based on the analytical performance of

the assay and CDC recommendations, with a Ct of 38 used for swab samples and a Ct of 40 for tissue samples.

Viral isolation and titration

Tissue samples with Ct values below 28 were selected for virus isolation and titration following preparation of clarified 10% (w/v) tissue homogenates. For virus isolation, 10^{-2} dilution of tissue homogenates were inoculated in duplicates to individual wells of 24 well plates seeded with Vero-E6 cells, followed by gentle rocking to ensure even distribution of the inoculum. After a 2-hour incubation at 37 °C, Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution was added to each well, and cultures were incubated at 37 °C in 5% CO_2 for 4 days. The culture medium was then removed and the cells were fixed with 80% acetone for 12 minutes. Next, rabbit anti-vaccinia virus (VACV) polyclonal antibody (PA1-7258, Invitrogen, Waltham, MA, USA) diluted in 1% horse serum albumin (HSA) in PBS was added and incubated for 1 hour at room temperature. Plates were washed twice with PBS-T (0.05% Tween-20) and once with PBS, after Alexa Fluor 488 goat anti-rabbit IgG (1:1000 in 1% HSA in PBS; Invitrogen) was added and incubated for 1 hour at room temperature. Plaques were then visualized using an EVOS fluorescence microscope.

Viral titers in tissue homogenates were determined using a plaque assay on Vero E6 cells. Tissue homogenates were subjected to serial 10-fold dilutions and dilutions ranging from 10^{-2} to 10^{-4} were inoculated in duplicate onto Vero E6 cell monolayers in 12-well plates. Following a 1-hour adsorption period, an overlay consisting of 0.8% methylcellulose prepared in DMEM supplemented with 2% FBS was added to each well. Cultures were incubated at 37 °C in 5%

CO_2 for 3 days. Following incubation, the methylcellulose overlay was removed, and the cell monolayers were fixed and stained for 1 hour using a solution containing crystal violet, formaldehyde, ethanol, and acetic acid. The plates were then washed with water and visible plaques were enumerated. Viral titers were quantified as plaque-forming units per gram of tissue (PFU/g), accounting for the initial 10% (w/v) tissue homogenate, dilution factor, and inoculum volume.

ELISA

Blood was collected from mice by terminal cardiac puncture under anesthesia at the study endpoint, which were allowed to clot and then centrifuged at 3,000 rpm for 10 minutes to obtain serum, which was stored at −80 °C. On the day of the assay, serum was heat-inactivated at 60 °C for 20 minutes and transferred from BSL-3 to BSL-2. Anti-MPXV IgG was analyzed using a commercial ELISA kit (Abbexa, catalogue no. abx392533) according to the manufacturer's instructions. Briefly, a 96-well plate was washed twice, after which 50 µL of serially diluted standards, blanks, and 1:20 diluted samples were added in duplicate. The plate was incubated at 37 °C for 30 minutes and then washed three times. Next, 50 µL of detection reagent was added and incubated at 37 °C for 30 minutes, after which the plate was washed five times. TMB substrate was added and incubated at 37 °C for 10 minutes in the dark, and the reaction was stopped with stop solution. Absorbance was measured at 450 nm using an ELISA plate reader. The positive cutoff was defined as the mean absorbance plus 3 SD of control animals on the same plate. Anti-MPXV IgG concentrations in positive serum samples were determined by interpolation from the standard curve and multiplied by the dilution factor.

Statistical analysis

Data from the study were analyzed and graphs were generated using Microsoft Excel (Microsoft 365), GraphPad Prism version 10.6.1 (892) and R version 4.6.1. Body weights were expressed as a percentage of each animal's day 0 (pre-inoculation) weight. Differences in percentage body weight change between inoculation groups were assessed by two-way repeated-measures ANOVA, followed by Tukey's multiple comparisons test. Differences in percentage body weight change between male and female mice within each inoculation group were assessed by two-way repeated-measures ANOVA, followed by Šídák's multiple comparisons test. Clinical scores were compared between male and female mice within each inoculation group by two-way ANOVA with Tukey's multiple comparisons test. Mean Ct values were compared among tissue types by the Friedman test with Dunn's multiple comparisons test. Data are presented as mean ± SEM, and differences were considered significant at $P < 0.05$.

Results

Mucosal inoculation leads to minimal overt clinical disease in CAST/EiJ mice

CAST/EiJ mice were infected with MPXV clade IIb via different routes, including mucosal inoculation. Clinical observations and body weight measurements were recorded daily to assess disease progression and morbidity following virus inoculation. Sham-infected mice exhibited steady weight gain and no clinical signs throughout the study period (Figure 3.2 and 3.3).

Male mice infected via the intraperitoneal route began to lose body weight around 4 days post-inoculation (dpi), with the group mean showing an approximate 5% reduction by 7 dpi (Figure 3.2). Within group, two of five mice experienced more pronounced weight loss, reaching approximately 8–10% for a brief period before recovery. Body weight loss in male mice at 7 dpi and 9 dpi was significantly greater than that observed in male control mice ($P = 0.0231$ at 7 dpi; $P = 0.0439$ at 9 dpi). The group mean body weight recovered to baseline levels by around 10 dpi, although a few individual mice required additional days before resuming consistent weight gain.

In contrast, female mice inoculated by the same route did not experience loss in body weight, however, showed a period of stagnant weight gain for several days before resuming their normal weight gain at 10 dpi, coinciding with recovery period in male (Figure 3.2). Despite the differing trends in body weight loss and clinical signs between the sexes, no statistically significant difference in weight change was observed between the male and female mice within the i.p. group except at 2 dpi ($P = 0.0422$).

Clinical progression was monitored using a predefined scoring system that assessed general health, clinical signs and lesion development following infection. Four parameters were evaluated: appearance, activity, breathing, and lesion formation (Table 3.1). Beginning at 4 dpi, mice infected via the intraperitoneal route developed mild clinical signs, initially characterized by reduced activity, followed over subsequent days by additional signs such as ruffled fur and labored breathing, with peak clinical scores observed at 7 dpi and subsequent quick recovery to normal by approximately 10 dpi (Figure 3.3). The clinical presentation closely paralleled the period of body weight loss, supporting the association between clinical disease severity and weight loss in this model. Female mice in this group exhibited milder clinical manifestations, with clinical scores significantly lower than those of males from 4 to 9 dpi ($P < 0.05$; two-way

ANOVA with Tukey's multiple comparisons test). Only a subset of females showed transient reduced activity for two days between 6 and 8 dpi, after which they returned to normal behavior. No skin lesions were observed in either sex during the study period.

Mice were infected via three different mucosal routes: intranasal, rectal, and genital. Following intranasal inoculation, male mice were slightly less affected compared to females (not statistically significant). Male mice did not develop appreciable body weight loss (Figure 3.2). A marginal decrease of about 1.5% at 7 dpi was observed; however, their weight gain remained stagnant until around 10 dpi, after which steady weight gain was observed. Female mice in this group exhibited body weight loss starting 6 dpi that persisted for three days; animals regained their original weight by 9 dpi and began to gain weight thereafter. In this group, both male and female mice followed a broadly similar pattern of body weight change and clinical presentation. No overt signs of mpox or cutaneous lesions were observed; however, subdued activity was noted in both sexes at 7 dpi (Figure 3.3).

During the clade IIb mpox outbreak, sexual contact was a major route of transmission, with characteristic anogenital lesions. In this study, mice of both sexes were infected via rectal and genital routes to investigate these inoculation pathways. Animals in these groups showed consistent body weight gain over the course of the study (Figure 3.2). They remained bright and alert with no signs of general malaise, except for rectally infected females that showed slightly reduced activity at 7 dpi (Figure 3.3). No overt clinical signs or cutaneous lesions characteristic of mpox were observed in any group.

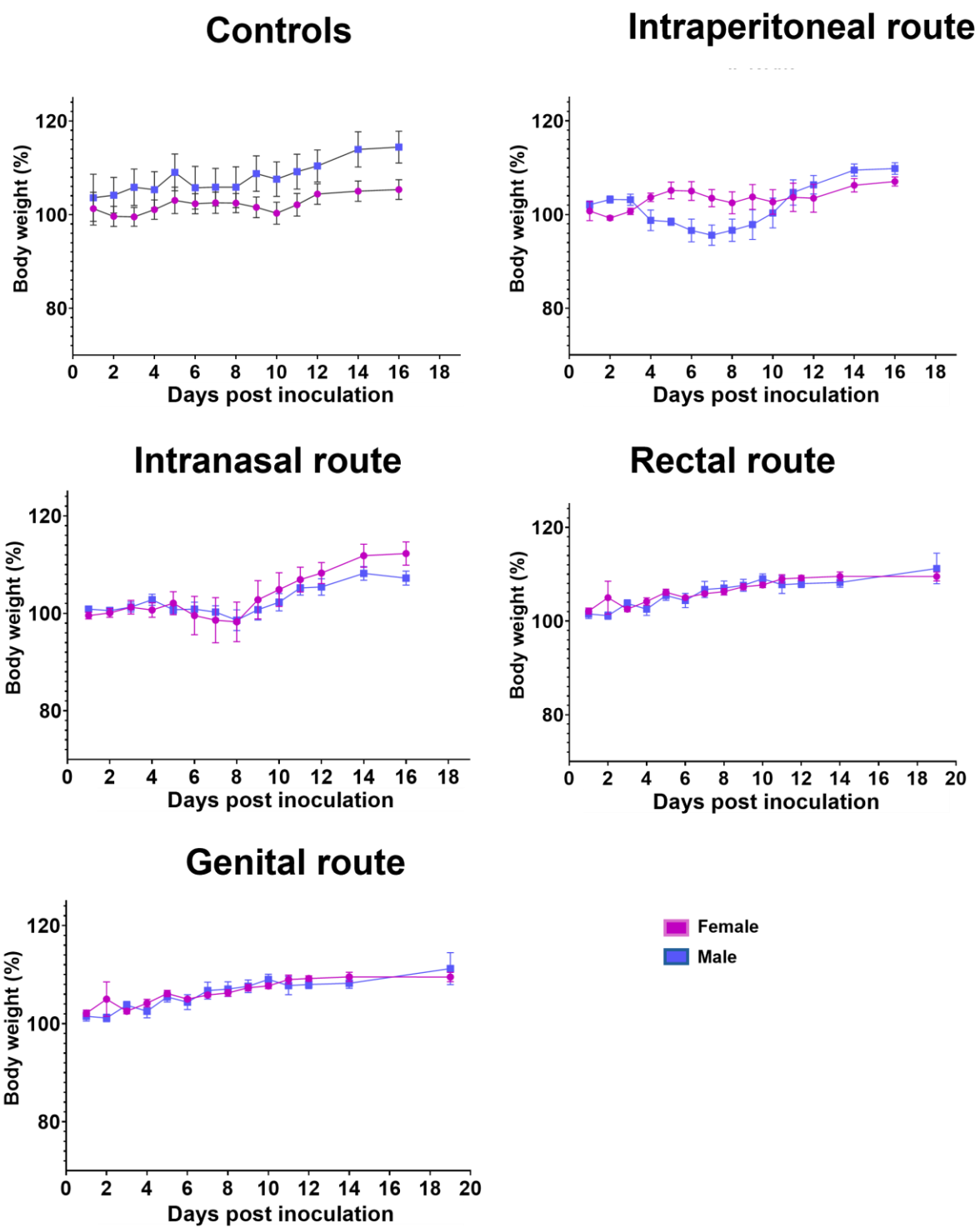


Figure 3.2 Longitudinal body weight changes following MPXV clade IIb inoculation.

Percent body weight changes relative to baseline for male and female mice (n = 5 per sex per group) following sham infection (controls), intranasal (i.n.), intraperitoneal (i.p.), rectal and

genital inoculation with MPXV clade IIb. Data points represent group means and error bars indicate the standard error of the mean (SEM).

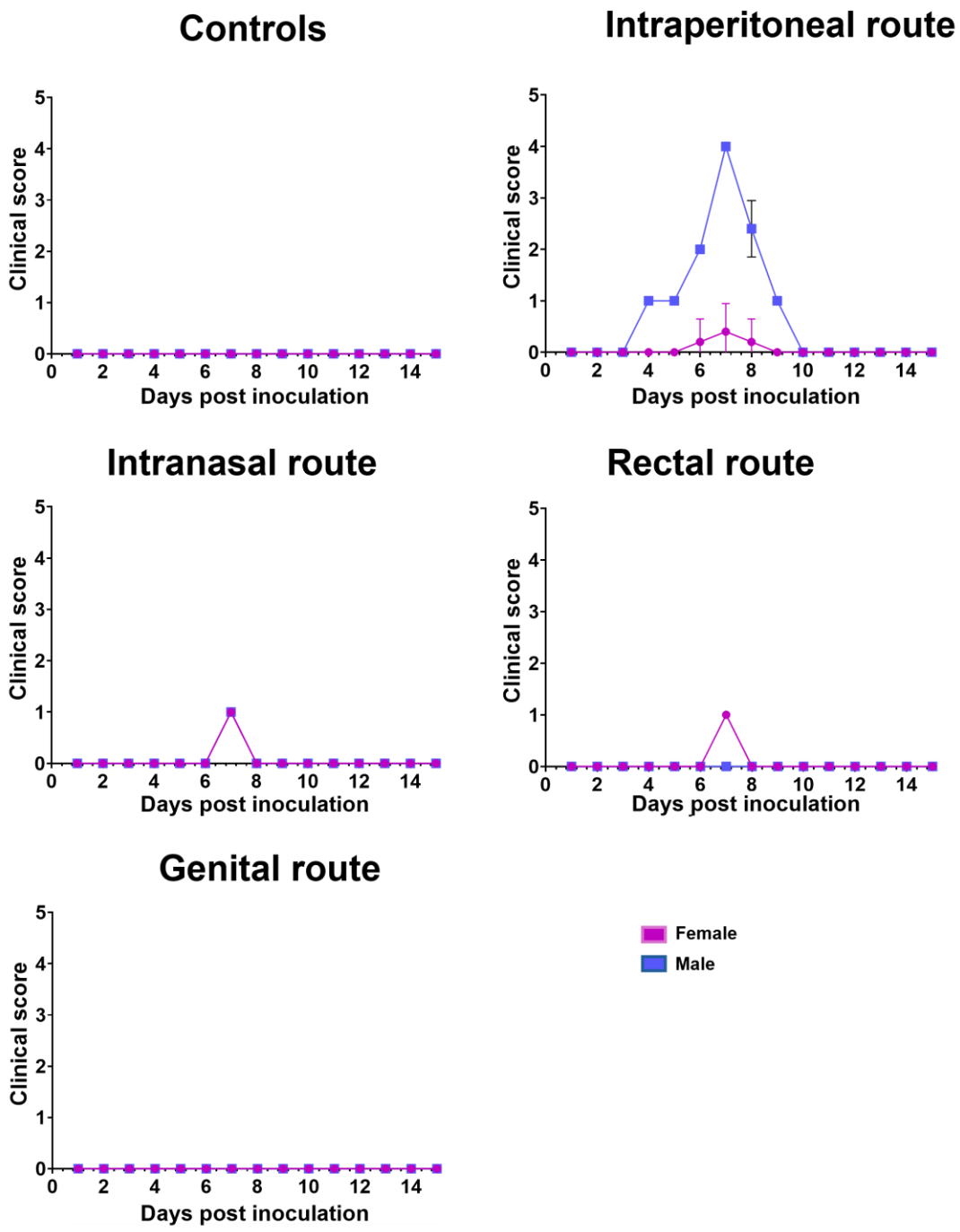


Figure 3.3 Clinical score following MPXV clade IIb inoculation. Clinical scoring of disease severity based on a cumulative assessment of four parameters: physical appearance, behavioral activity, respiratory effort and skin lesions. Data represent mean clinical scores for male and female mice (n = 5 per sex per group) following sham infection (controls), intranasal (i.n.), intraperitoneal (i.p.), rectal, and genital inoculation with MPXV clade IIb. Error bars indicate standard error of the mean (SEM).

Sustained recovery of viral DNA at mucosal inoculation sites

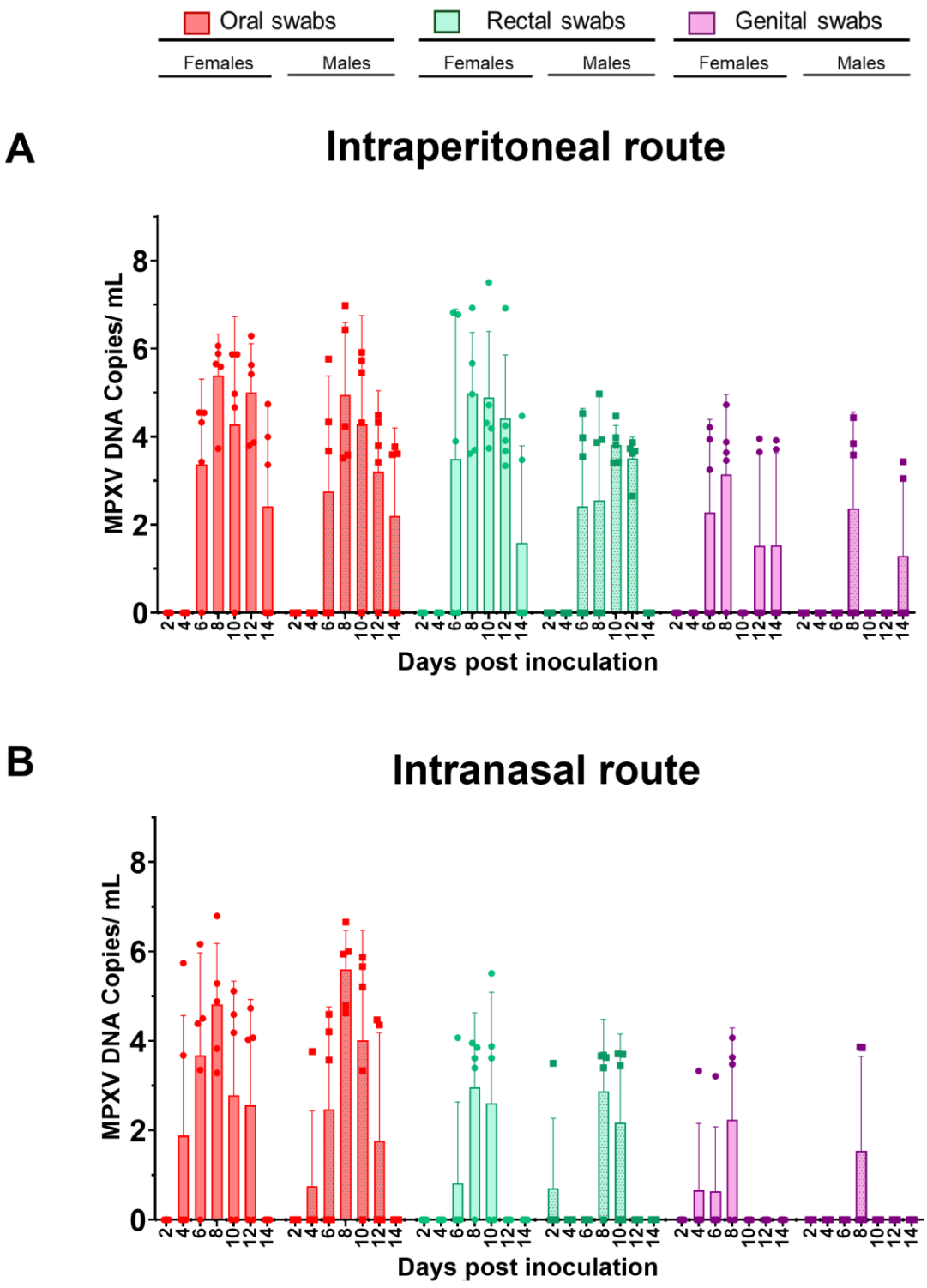
Oropharyngeal, rectal and genital swabs were collected from mice at regular intervals and viral DNA in swabs were quantified by qPCR. Viral DNA was detected in oral swabs of intraperitoneally infected mice beginning at 6 dpi, with levels peaking around 8 dpi, and remained detectable through the final sampling time point at 14 dpi (Figure 3.4). A similar shedding pattern was observed in rectal swabs from this group. Sustained detection of viral DNA was evident for at least 4 days, starting from the earliest detection time point at 6 dpi. Both male and female mice exhibited comparable oropharyngeal shedding patterns despite differences in clinical morbidity. Detection of viral DNA in genital swabs from intraperitoneally infected mice was sporadic and inconsistent across the sampling period.

Following intranasal infection, viral DNA was detectable in oropharyngeal swabs starting at 4 dpi (Figure 3.4). Shedding peaked at 8 and 10 dpi and declined thereafter, with viral DNA no longer detectable after 12 dpi. Mice exhibited only minimal viral DNA shedding in rectal and genital swabs, with low-level detection primarily around 8 dpi.

Mice infected via rectal inoculation exhibited high levels of viral DNA shedding in rectal swabs. Viral DNA was detected as early as 2 dpi (the first day of swab collection) and remained

detectable through the final sampling time point (Figure 3.4). Shedding peaked at 8 and 10 dpi, with the highest mean viral DNA copy numbers observed on these days. Low levels of viral DNA were also detected in genital and oral swabs at time points following peak rectal shedding, which may reflect self-grooming behaviors leading to mechanical transfer of virus-laden material from the perianal region to other sites.

Within the genital inoculation group, there was greater variability in viral DNA shedding among males and females. In male mice, shedding patterns were inconsistent across individuals and sample types. Inoculation via the preputial route is challenging, making it difficult to achieve consistent infection across mice. The small genital opening, resistance in uptake of the inoculum and technical difficulties during administration are factors that may contribute to the observed variability. In contrast, female mice infected via the vaginal route showed high viral DNA copy numbers in genital swabs as early as 2 dpi, with levels increasing until 8 dpi and remaining detectable throughout the sampling period (Figure 3.4). Rectal swabs from these females displayed a similar pattern, with earliest detection at 2 dpi, peak shedding around the same time as genital swabs, and sustained high-level detection over an extended period. Additionally, viral DNA was recovered from oral swabs for a prolonged period in a subset of animals. The detection of high levels of viral DNA at sites distant from the primary inoculation site could suggests possible secondary spread along mucosal surfaces and/or transfer through grooming.


Oral swabs
Rectal swabs
Genital swabs
Females
Males
Females
Males
Females
Males
A
Intraperitoneal route
MPXV DNA Copies/ mL
Days post inoculation
B
Intranasal route
MPXV DNA Copies/ mL
Days post inoculation

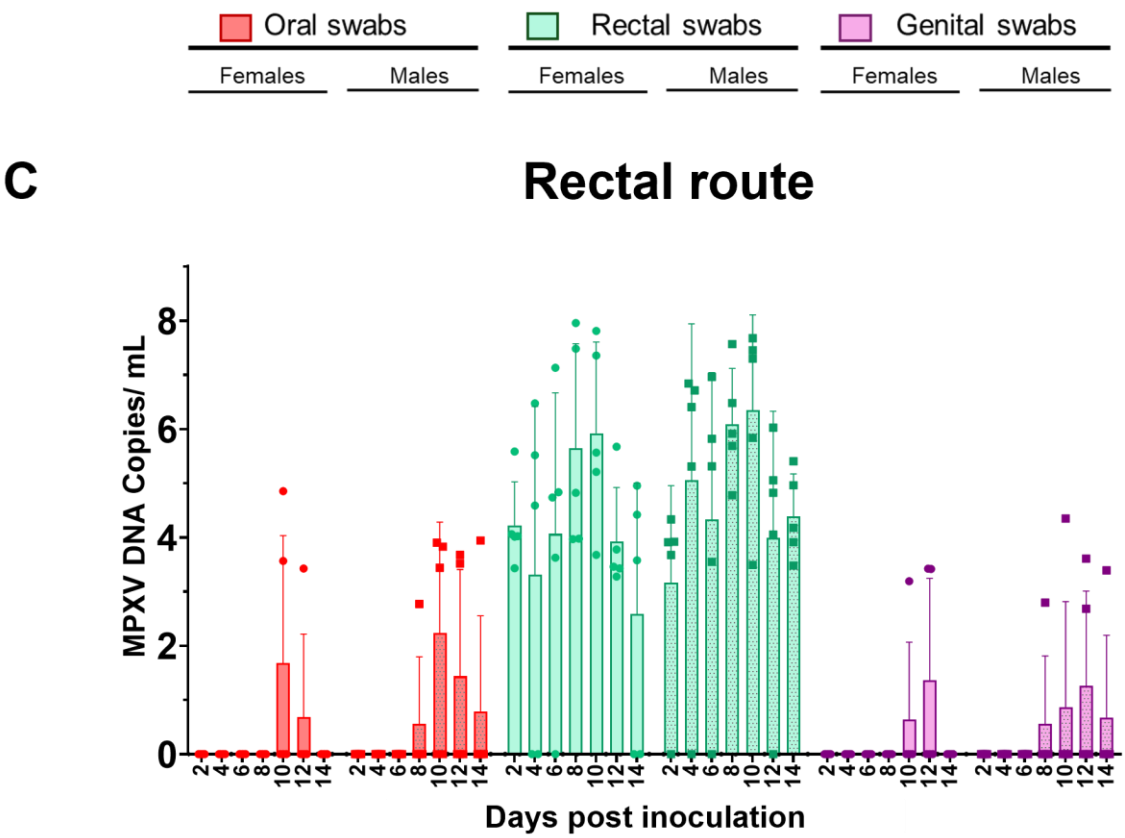

Oral swabs
Rectal swabs
Genital swabs
Females
Males
Females
Males
Females
Males
C
Rectal route
MPXV DNA Copies/ mL
Days post inoculation
D
Genital route
MPXV DNA Copies/ mL
Days post inoculation

Figure 3.4 Viral DNA shedding in swabs following MPXV IIb inoculation. MPXV DNA copy numbers, presented as $\log_{10}$ copies per milliliter (Y-axis), for longitudinally collected swab samples from oral (red), rectal (green) and genital (purple) sites in female and male mice (n = 5 per sex per group). Panels represent viral shedding following: (A) intraperitoneal (i.p.), (B) intranasal (i.n.), (C) rectal, and (D) genital inoculation with MPXV clade IIb. Copy numbers were calculated using a standard curve generated from MPXV standards. Bars represent mean viral DNA copy numbers and error bars indicate standard deviation (SD). Abbreviations: F; female, M; male.

Limited detection of viral DNA in tissues at necropsy following mucosal inoculation

Tissues were collected during the necropsy to evaluate the replication of viruses at site of inoculation and dissemination to other organs. MPXV after initial replication at site of inoculation are relocated via tissue-resident immune cells to nearby draining lymph nodes, where infection is established, followed by dissemination through the hematolymphatic system into secondary organs. Liver, spleen, lungs, kidney, colon, rectum and reproductive organs were collected and viral DNA were detected by qPCR.

Viral DNA was detected in all tissue types collected from mice infected via the intraperitoneal route (Figure 3.5). In both sexes, MPXV DNA was detected at higher levels in the reproductive tract than in other tissues (significant versus liver, spleen, and rectum, $P < 0.05$; Friedman test with Dunn's multiple comparisons, sexes combined). Tissue samples with Ct values below 28 were subjected to virus isolation and titration. Infectious virus was recovered only from the reproductive organs of two male mice infected intraperitoneally, at titers of 2.53×10^5 and 5.50×10^4 PFU/gram of tissue.

In mice infected via the intranasal route, viral DNA was recovered from the lungs of most animals at low levels, with no detection in other organs except the colon of one mouse (Figure 3.5). In the rectal inoculation group, viral DNA was detected only sporadically and at very low levels in the colon, rectum, liver and spleen. In female mice infected via the vaginal route, viral DNA was detected in the rectum (3/5 mice) and kidney (1/5), in addition to the reproductive organs (1/5). No viral DNA was recovered from any organs in the male genital inoculation group (Figure 3.5). Positive tissue samples from all mucosal groups had Ct values above 35, indicating low-level detection.

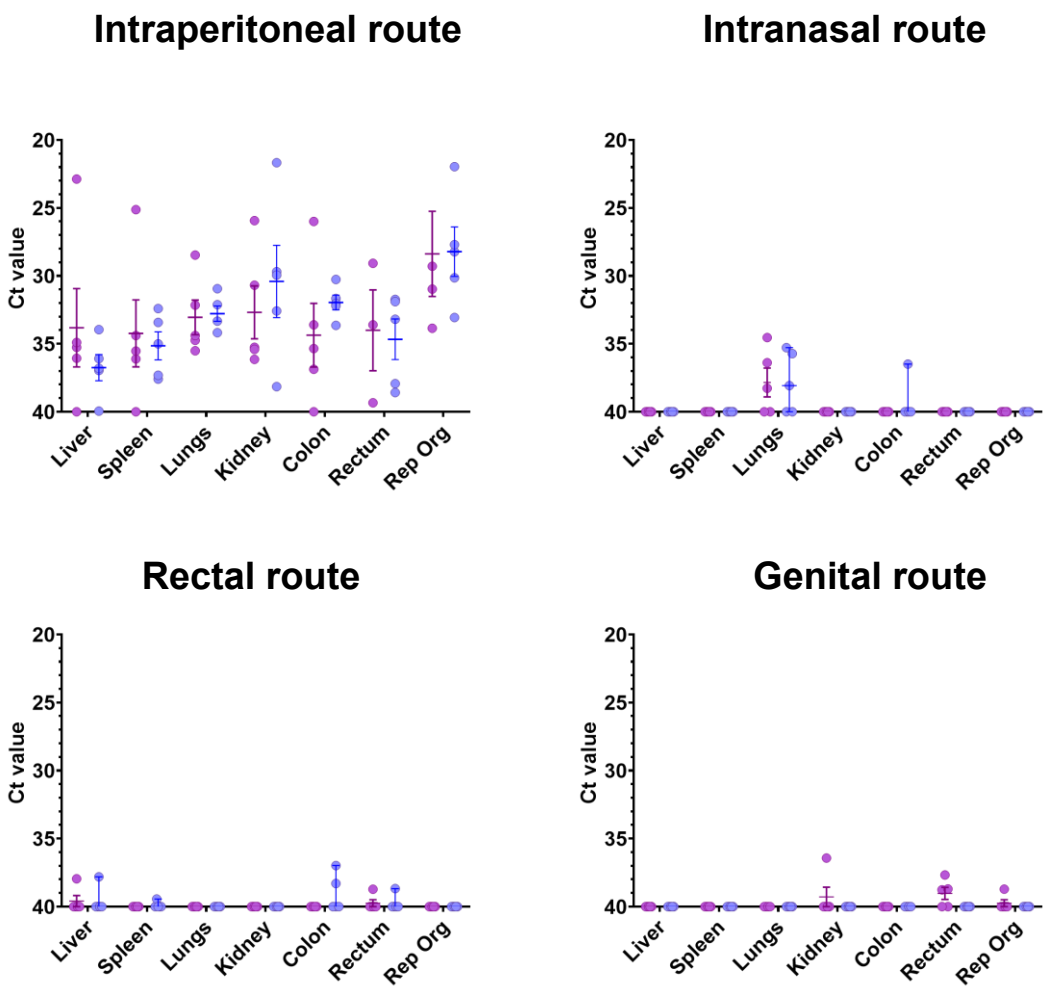


Figure 3.5 MPXV DNA detection in tissues following MPXV IIb inoculation. Cycle threshold (Ct) values from qPCR analysis of tissues collected at the study endpoint from mice inoculated by the intraperitoneal (i.p.), intranasal (i.n.), rectal and genital routes. Each symbol represents an individual animal; bars indicate the mean Ct and error bars the standard deviation (SD).

Route-dependent seroconversion following mucosal inoculation

Serum collected from blood at study endpoint was evaluated for anti-MPXV IgG using the commercial ELISA kit. Following intraperitoneal inoculation, all five males and all five females had detectable anti-MPXV IgG, with mean IgG levels for both sexes close to the upper limit of detection, indicating seroconversion in all inoculated mice. A similar result was observed in the intranasal group, in which all five males and all five females seroconverted, with IgG detected at higher levels in the majority of animals. In the rectal group, two of five females and four of five males seroconverted. Three of five females inoculated via the genital route seroconverted, with anti-MPXV IgG concentrations above the upper limit of quantification. None of the males in the genital group seroconverted. No control animal was seropositive; all control sera fell below the limit of quantification.

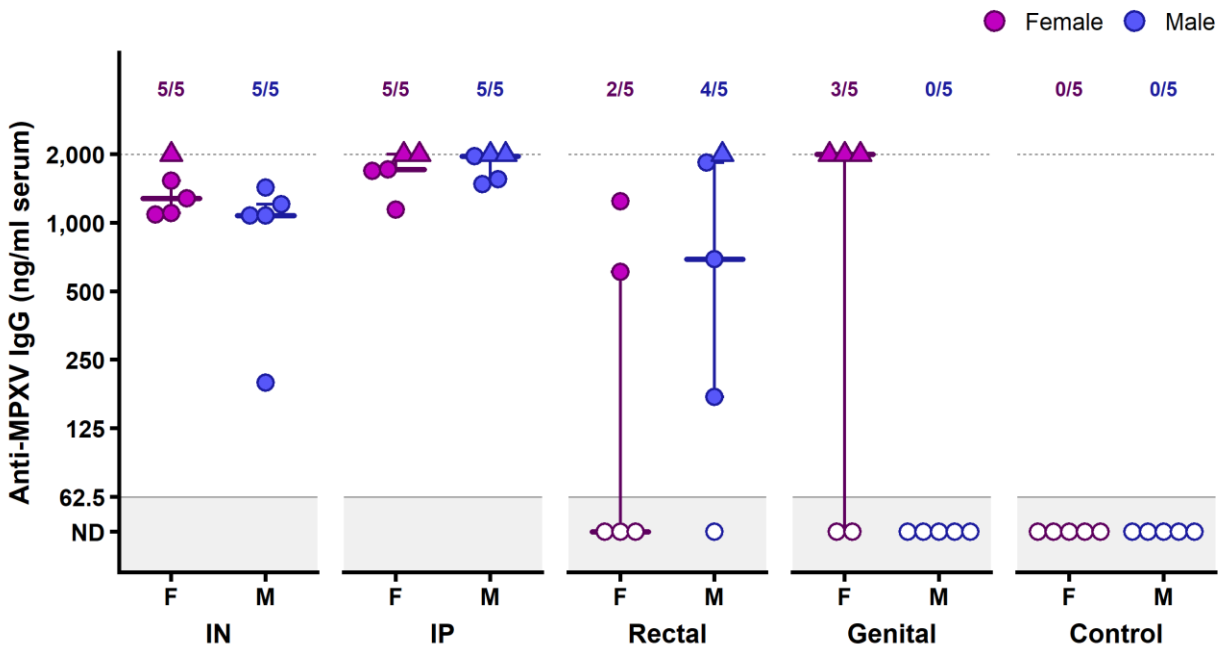


Figure 3.6 Anti-MPXV IgG responses in serum following MPXV clade IIb inoculation. Serum collected at study endpoint was assayed for anti-MPXV IgG by commercial ELISA. Results are shown for female (magenta) and male (blue) mice (n = 5 per sex per group) following intranasal (i.n.), intraperitoneal (i.p.), rectal, and genital inoculation, or sham inoculation (controls). Each symbol represents an individual animal; bars indicate the median and whiskers the interquartile range. Numbers above each group indicate the number of seropositive animals out of the total. The shaded band denotes values below the lower limit of quantification (LLOQ, 62.5 ng/mL), with open circles indicating samples in which anti-MPXV IgG was not detected (ND). The dashed line denotes the upper limit of quantification (ULOQ, 2,000 ng/mL), and triangles indicate samples with concentrations above the ULOQ. The seropositivity cutoff was defined as the mean absorbance plus 3 SD of control animals on the same plate.

Discussion

The 2022 global outbreak caused by MPXV clade IIb, a newly emergent subclade, is the largest mpox outbreak in history in terms of magnitude and geographical coverage. Clade IIb presents novel and unique epidemiological features, particularly regarding transmission dynamics and clinical presentation compared to historical outbreaks. Historically, outbreaks were sporadic and caused by zoonotic spillover of MPXV in rainforest communities of endemic African regions through wildlife contact. In the 2022 outbreak, transmission was predominantly driven by intimate sexual contact within MSM communities[55]. This shift in transmission dynamics necessitates development of new mpox models involving intimate contact transmission routes to investigate the underlying pathogenesis associated with this novel route of infection.

Classical inbred mice are resistant to MPXV infection. Americo et al. in 2010, after screening 37 commercial mouse strains for MPXV susceptibility, discovered CAST/EiJ mice to be susceptible to MPXV infection and subsequently developed and evaluated this mouse strain as a suitable small animal mpox model[178]. Since then, CAST/EiJ mice have been extensively used in mpox research involving different routes including intraperitoneal, intranasal, intradermal, and so on[33,179,187,200,201]. However, experimental studies examining MPXV infection via rectal and genital mucosal routes remain limited, and the development of a small animal model to better understand the underlying pathogenesis involving mucosal routes is critical. Here, we infected CAST/EiJ mice via four different routes, intraperitoneal, intranasal, rectal, and genital, with the objective of investigating the susceptibility of CAST/EiJ mice to MPXV clade IIb infection following mucosal inoculation. Because earlier reports suggest that the

intraperitoneal route produces more severe disease compared to other routes[178,179], it was used as the positive control against which the mucosal routes were compared.

In our study, intraperitoneal inoculation of MPXV clade IIb at 10^5 PFU was non-lethal and resulted in moderate disease with slight weight loss and systemic clinical signs. The virus disseminated into internal organs, all inoculated mice seroconverted, and viral shedding was found consistently in oral and rectal swabs. Americo et al. also reported no weight loss or lethality in intraperitoneally infected CAST/EiJ mice at doses up to 10^5 PFU of MPXV clade IIb. However, clade Ia and IIa had LD_{50} values of 1 PFU and 10^3 PFU respectively when infected via same route[179]. The marked attenuation of clade IIb virulence in CAST/EiJ mice has been reported in other studies and is consistent with the milder clinical severity observed during the 2022 global outbreak. The enhanced human transmission coupled with virulence attenuation in clade IIb raises the possibility of adaptation of MPXV to humans with a concomitant loss of fitness in other host species[33,55,201]. Similarly, intranasal inoculation of CAST/EiJ mice with MPXV clade IIb in our study resulted in no clinical signs or body weight loss, consistent with reports from other studies, where the newly emerged clade was similarly reported to be attenuated via this route[179,201].

Infection of CAST/EiJ mice with MPXV clade IIb via rectal and genital routes resulted in asymptomatic infection, with no signs of disease, skin lesions or weight loss. However, viral DNA was consistently detected over a sustained period at the site of inoculation. This sustained shedding of viral DNA, together with seroconversion in a proportion of inoculated animals, suggests active viral replication at the respective mucosal site. This is consistent with prior reports of pox-associated pathology and detectable viral antigen and RNA in rectal and vaginal lesions in nonhuman primate and rodent model, supporting active mucosal viral replication[204,245].

Port et al. used the multimammate rat (*Mastomys natalensis*) as a model for MPXV mucosal transmission, infecting rats with a 2022 human outbreak MPXV isolate via different routes, including mucosal inoculation. Rectal and vaginal inoculation in these rats resulted in no clinical signs of disease, no weight loss and no skin lesions, but a high viral burden was detected in oral, rectal, and urogenital swabs, consistent with the findings from our study. However, following rectal and genital inoculation in multimammate rats, virus was detected in multiple internal organs, including the lungs, liver, and spleen at day eight post-infection, suggesting systemic viral spread[204]. In contrast, in our study, viral DNA was not recovered from internal organs in the rectal or genital inoculation groups. Since organs were collected only at the study endpoint, it remains possible that virus was disseminated to internal organs but was efficiently cleared by that time point. To address this, we conducted a serial sacrifice study to map viral dissemination from the initial site of mucosal inoculation over time (study not yet published).

Studies in nonhuman primates using rectal mucosal inoculation with high viral doses (greater than 10^6 PFU) have produced clinical symptoms such as localized proctitis, rectal exudate, weight loss, and anogenital skin lesions, along with rectal and genital shedding of the virus. Disease progression, clinical symptoms and lesions, and overall prognosis in NHPs were more reflective of human mpox disease compared to other animal models. However, the housing and handling requirements of large animals under biocontainment, along with high cost and limited access to research animals, remain major limitations of this model[162,245].

Anorectal viral shedding was commonly reported during the 2022 mpox outbreak. In fact, anorectal swabs ranked second only to lesion swabs in terms of PCR positivity for MPXV DNA among mpox patients in this outbreak[263]. In our study, rectal shedding of viral DNA was consistently detected, beginning early during infection, in mice infected via the i.p., rectal, and

genital routes. Similar findings have been reported in experimental studies using rat and NHP models[162,204,245]. For mpox diagnosis during outbreaks, lesion swabs remain the most commonly collected sample. However, in the 2022 outbreak, lesions were less frequently reported overall and most lesions were concentrated in the anogenital region, an area that is less accessible for examination, less often reported by patients and frequently kept concealed due to stigma[264,265]. In such cases, rectal swabs, which can be self-collected by patients, may serve as an important sample type for early diagnosis.

Our findings suggest that CAST/EiJ mice could serve as a model for studying the underlying pathogenesis of MPXV infection associated with intimate sexual contact in humans. Although MPXV clade IIb infection via the rectal and genital routes in CAST/EiJ mice produced no rash, cutaneous lesions or other overt clinical features of mpox, we observed active, sustained viral replication at the local mucosae with prolonged, high-level viral shedding. CAST/EiJ mice could therefore be developed as a transmission model for MPXV rather than a lethality model, which better correlates with the current epidemiology of mild, self-limiting disease with efficient human-to-human transmission. This model could further be used to evaluate the immune response, transmission dynamics, the efficacy of vaccines and antivirals in preventing mucosal infection and shedding. Similarly, infection with other viral clades via mucosal routes will help answer whether the enhanced mucosal replication and shedding observed here is specific to clade IIb, possibly attributed to recent mutations associated with this novel clade's adaptation to human hosts or whether it is a shared feature across other MPXV clades.

There are several limitations to this study. First, only one clade of virus was used, so further evaluation with other clades is needed. Second, whether viral shedding results in efficient transmission to sentinel animals was not analyzed in this study. Similarly, the high inoculation

dose and the method of virus administration (using a pipette and tips) may not accurately reflect human exposure during sexual or intimate contact. Inoculation in this study was performed on intact mucosa, which may not fully correlate with the microtrauma often involved in mucosal transmission in humans.

Chapter 4 - Conclusion

Mpox continues to circulate in both endemic and non-endemic regions, with no clear signs of retreat. Following the 2022 global outbreak, another outbreak caused by the newly emerged subclade Ib in the DRC in 2023, together with travel-associated cases exported to multiple countries, prompted the WHO to declare a Public Health Emergency of International Concern for a second time. Both outbreaks, driven by distinct subclades yet sharing novel transmission patterns, epidemiological features, and atypical clinical presentations, raised new risks and questions that remain to be fully addressed.

The more widely and persistently MPXV circulates in humans, the greater the opportunity for the virus to encounter, infect, and adapt to novel animal hosts. The first study in this thesis addressed this concern by investigating the susceptibility of cattle, a species maintained in close contact with humans and permissive to other related orthopoxviruses, to MPXV clade IIb. Our results suggest that cattle are minimally susceptible, as calves remained subclinical with no overt signs of disease and supported only transient, localized mucosal replication without systemic dissemination, making the establishment of a persistent bovine reservoir unlikely. Although reassuring, these findings do not eliminate the broader risk. From documented spillover to animals such as prairie dogs in non-endemic regions, to the detection of MPXV in wild *Bovidae* in endemic regions, the host range of this virus remains incompletely defined. Continued surveillance of animal populations, together with experimental infection of vulnerable species, therefore remains essential to anticipate and contain future spillover and spillback events.

At the same time, human-to-human transmission continues, predominantly through intimate sexual contact, as seen in both the 2022 clade IIb and 2023 clade Ib outbreaks, yet little

is known about the mucosal transmission events central to this spread. The second study in this thesis aimed to address the need for an appropriate small animal model to investigate the mucosal pathogenesis and transmission of the disease. Here, we evaluated the susceptibility of CAST/EiJ mice to mucosal infection with MPXV clade IIb. Although mucosal inoculation did not produce overt disease, it established active, sustained viral replication and prolonged, high-level shedding at the inoculation sites. This model could therefore be developed as a mucosal transmission model that reflects the mild, self-limiting disease of the current outbreak rather than a lethality model. Further characterization is nonetheless needed; as a continuation of this work, we have undertaken serial-sacrifice studies to map viral dissemination following mucosal inoculation and studies with additional clades, with transmission and immune-response studies planned to further develop this model.

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Appendix-A: Supplementary file

Supplementary Table 1: MPXV DNA detection in tissues collected at necropsy from experimentally inoculated cattle.

Cycle threshold (Ct) values for MPXV DNA detected by real-time PCR (G2R-G assay) in tissues collected at necropsy from six principally infected calves (blue) and three sentinel calves (pink) at 7, 14, and 20/21 days post inoculation (dpi). The right-hand panel shows individual skin samples collected from each animal. Each value is the mean of duplicate reactions. Gray cells indicate samples not collected; white cells indicate samples not analyzed.

Abbreviations: N1, nasal turbinate (rostral); N2, nasal turbinate (caudal); IC junction, ileocecal junction; PTON, palatine tonsil; RPLN, retropharyngeal lymph node; ManLN, mandibular lymph node; CMLN, caudal mediastinal lymph node; TBLN, tracheobronchial lymph node; ICLN, inferior cervical lymph node; SCLN, superficial cervical lymph node; MesLN, mesenteric lymph node; GHLN, gastrohepatic lymph node; RecLN, rectal lymph node; IngLN, inguinal lymph node; PFLN, prefemoral lymph node; RetroLN, retroperitoneal lymph node; BM, bone marrow; Skin INC site, skin at inoculation site; dpi, days post inoculation; ND, not detected.

Sample Type	7 DPI		14 DPI		20/21 DPI				
	6129	6130	6126	6128	6137	6134	6135	6133	6127
Brain	ND	ND	ND	ND	ND	ND			
Olfactory bulb	ND	ND	ND	ND	ND	ND			
Eye lid (pool)	ND	ND	ND	ND	ND	ND			
N1	ND	ND	ND	ND	ND	ND			
N2	ND	ND	ND	ND	ND	ND			
Trachea	ND	ND	ND	ND	ND	ND			
Lung	ND	ND	ND	ND	ND	ND			
Heart	ND	ND	ND	ND	ND	ND			
Spleen	ND	ND	ND	ND	ND	ND			
Liver	ND	ND	ND	ND	ND	ND			
Kidney	ND	ND	ND	ND	ND	ND			
Small Intestine	ND	ND	ND	ND	ND	ND			
IC Junction	ND	ND	ND	ND	ND	ND			
Large Intestine	ND	ND	ND	ND	ND	ND			
Colon:Rectum	ND	ND		ND	ND	ND			
Tonsil	ND	ND	ND	ND	ND	ND			
PTON	ND	ND	ND	ND	ND	ND			
RPLN	ND	ND	ND	ND	ND	ND			
ManLN	ND	ND	ND	ND	ND	ND			
CMLN	ND	ND	ND	ND	ND	ND			
TBLN	ND	ND	ND	ND	ND	ND			
ICLN	ND	ND	ND	ND	ND	ND			
SCLN	ND	ND	ND	ND	ND	ND			
MesLN	ND	ND	ND	ND	ND	ND			
GHLN	ND	ND	ND	ND	ND	ND			
RecLN	ND	ND	ND	ND	ND	ND			
IngLN	ND	ND	ND	ND	ND	ND			
PFLN	ND	ND	ND	ND	ND	ND			
RetroLN	ND	ND	ND	ND	ND	ND			
BM	ND	ND	ND	ND					
Skin INC site	ND	ND	ND	ND	ND	ND			
Gum	ND	ND		ND	ND	ND			
Thymus	ND	ND							
Feces	ND	ND	ND	ND	ND	ND			
Skin									
Peyers Patch	ND	ND		ND	ND	ND			
Stomach	ND	ND		ND	ND	ND			
Esophagus	ND	ND		ND	ND	ND			

Sample Type (Skin)	7 DPI		14 DPI		20/21 DPI				
	6129	6130	6126	6128	6137	6134	6135	6133	6127
Skin	ND	ND	ND	ND		ND	ND	ND	ND
	ND	ND	ND	ND		ND	ND	ND	ND
	ND	ND	ND	ND		ND	ND	ND	ND
	ND	ND	ND	ND		ND	ND	ND	ND
	ND	ND	ND	ND		ND	ND	ND	ND
	ND	ND	ND	ND		ND		ND	
	ND	ND	ND			ND		ND	
	ND	ND	ND			ND		ND	
	ND	ND	ND			ND		ND	
	ND	ND	ND					ND	
	ND	ND	ND					ND	
	ND							ND	
	ND								
	ND								
	ND								
	ND								
	ND								

Legend	
	Principally infected animals
	Sentinels
	Samples not collected
ND	Not detected
	Not analyzed