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RESEARCH LETTER

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Characterization of monkeypox virus clade IIb lineage B1 strains in animal models: insights into virulence

Mpox is a generalized infection caused by the monkeypox virus (MPXV), a member of the *Poxviridae* family (genus: *Orthopoxvirus*) [1]. This zoonotic virus primarily affects small mammals such as squirrels, rats, and shrews in Africa, with occasional spillover to monkeys and humans [2,3]. While endemic in some African countries, MPXV has caused sporadic outbreaks, including global spread to non-endemic regions since May 2022 [4].

MPXV has a double-stranded DNA genome (~190 kb) divided into three regions: core, left, and right inverted terminal repeats (ITR) [5]. Evolution in poxviruses is driven by gene gain and loss in these terminal regions, as observed during the 2022 mpox outbreak [6,7]. Phylogenetically, MPXV is classified into two clades: Clade I, found in Central Africa, which is in humans more virulent and resembles smallpox, and Clade II, which has a lower fatality rate and is present in West Africa [8]. Clade II is further subdivided into IIa and IIb, with the 2022 outbreak predominantly involving Clade IIb (lineage B.1) and showing a fatality rate of less than 1% [9,10].

To study species specific susceptibility to MPXV, evaluate potential zoonotic reintroduction and classify *in vivo* pathogenesis of different clades, Swinhoe's striped squirrels (*Tamiops swinhoei*) ($n = 6$) were intranasally inoculated ($10^{6.7}$ $TCID_{50}$) with the German isolate MPXV-BY-IMB25241 ("Clade IIb IMB 2022"). Highly susceptible CAST/EiJ mice (*Mus musculus castaneus*) were used to compare Clade IIb IMB 2022 ($n = 7$) with RKI335 2022 (a Clade IIb variant with genomic rearrangements [11]) ($n = 9$) and a reference MPXV Clade IIa strain from Côte d'Ivoire ($n = 7$) (Appendix, Figure S1).

None of the six inoculated squirrels displayed clinical signs or experienced weight loss (Appendix, Figure S2(a)). Viral DNA was detected in oral swabs from three individuals, with only one squirrel consistently testing positive across three time points (Appendix, Figure S2(b)). Fecal samples contained minimal viral DNA (Appendix, Figure S2(c)). Immunofluorescence assay confirmed antibodies in only two squirrels, suggesting productive infection in only a subset of cases (Appendix, Table S1). Therefore, it seems that Swinhoe's striped squirrels do not represent a productive host for MPXV, leading to an overall low risk assessment for anthropozoonotic respectively zoonotic infections from such species.

For clade-specific virulence testing [12], CAST mice were inoculated with Clade IIb IMB 2022, Clade IIa Côte d'Ivoire 2012, or human MPXV RKI335 (a German isolate with genome deletions from late 2022) with comparable infection doses of $10^{5.6}$ $TCID_{50}$, $10^{4.6}$ $TCID_{50}$, and $10^{5.5}$ $TCID_{50}$, respectively. Clade IIa Côte d'Ivoire 2012 exhibited the highest virulence, with a 20% body weight loss and a 71% survival rate, as two mice succumbed to the infection (Figure 1(a and b)). In contrast, Clade IIb IMB 2022 showed the lowest virulence, with all mice surviving without clinical signs and maintaining body weights above 90% of baseline (Figure 1(a and b)). The RKI335 mutant strain displayed intermediate virulence, with an 89% survival rate and weight loss not exceeding 10% (Figure 1(b)). Viral DNA was undetectable in oral swabs from Clade IIb IMB 2022 mice, whereas six of seven Clade IIa-infected mice and one RKI335-infected mouse tested positive (Figure 1(c)). Tissue analyses confirmed viral loads in organs of RKI335-infected mice, and Clade IIa-infected mice, while no viral DNA was detected in Clade IIb-infected mice (Figure 1(d)). Due to the number of animals, the statistical comparison of the groups could only be analyzed for the final time point at which there was no significant difference between the groups (Figure 1(d)).

Clade IIb IMB 2022 induced seroconversion in all inoculated mice, as measured by immunofluorescence, but no transmission to direct-contact cage mates (Appendix, Table S1). Clade IIa Côte d'Ivoire 2012 induced immune responses in nearly all mice as well, except for one, which succumbed at 7 dpi (Appendix, Table S1). All but one inoculated mouse of the RKI335 group (with characteristic deletions) seroconverted with additional evidence of transmission, as both direct-contact cage mates also tested positive for antibodies (Appendix, Table S1). These

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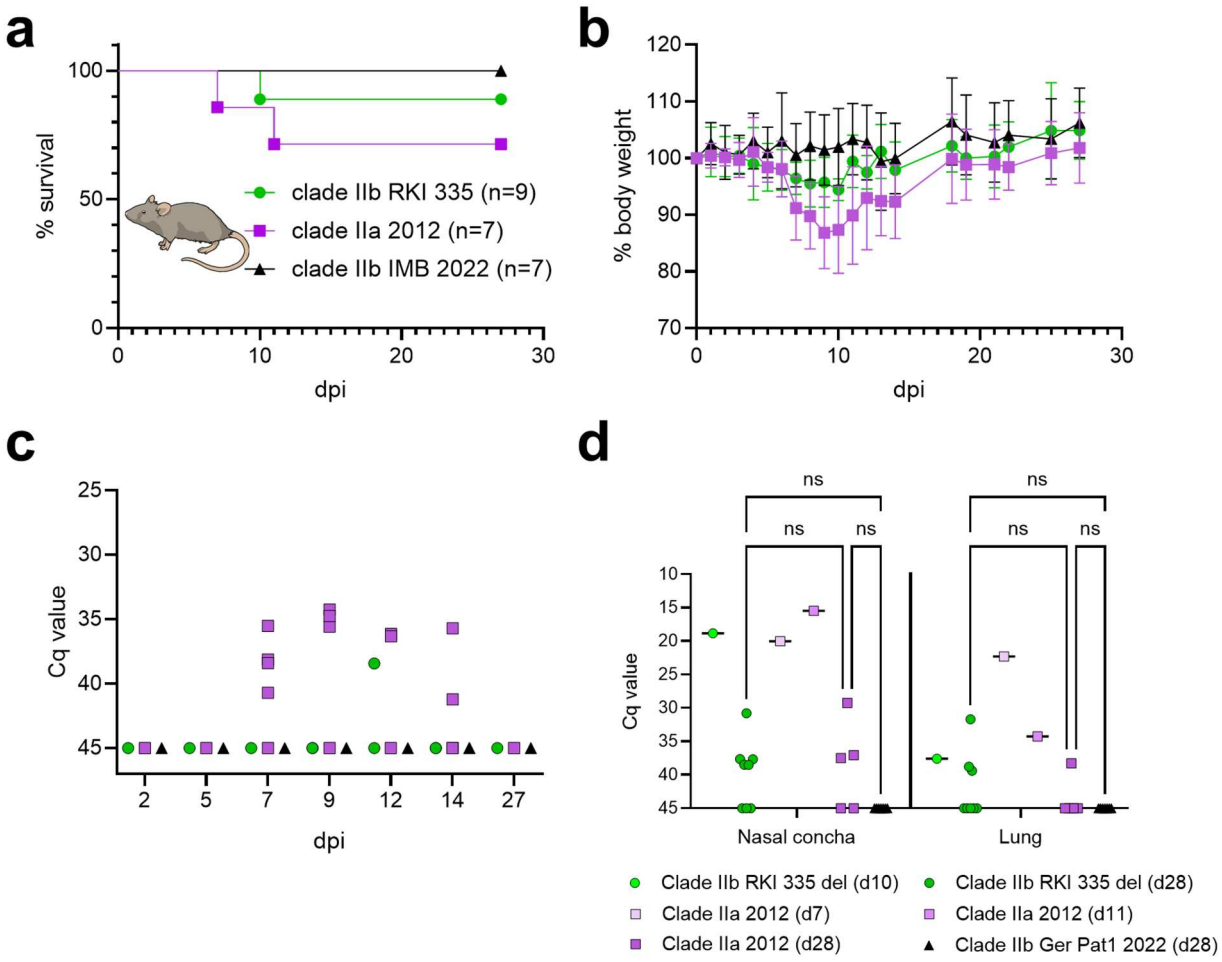

Figure 1. Virulence of MPXV "clade IIb RKI335" ($10^{5.5}$ $TCID_{50}$), "clade IIb IMB 2022" ($10^{5.6}$ $TCID_{50}$) and "clade IIa Côte d'Ivoire 2012" ($10^{4.6}$ $TCID_{50}$) upon intranasal inoculation of CAST mice. CAST mice were infected and the (a) survival and (b) percentage of starting weights are indicated. Bars indicate SD. (c) Buccal swab samples were analyzed for viral DNA and Cq-values were individually recorded. (d) Viral DNA detection by qPCR from lung and nasal turbinate samples collected at final endpoint from individual mice. Individual final days are indicated in the legend in parentheses. Two way Anova analysis was performed for groups with more than three individual samples. (NIAID Visual & Medical Arts. (7 October 2024). Lab Mouse. NIAID NIH BIOART Source. bioart.niaid.nih.gov/bioart/279).

results further support a virulence gradient in CAST mice, with Clade IIa being the most virulent, followed by the RKI335 deletion strain, and Clade IIb being the least virulent.

Discussion

Studying MPXV pathogenesis in potential reservoir hosts remains challenging due to uncertainties about relevant species. A widely accepted animal reservoir model for MPXV is lacking, yet identifying natural reservoirs is crucial for understanding spillover risks to humans. Some squirrel species are considered potential reservoirs, prompting our experimental inoculations in *Tamiops swinhoei*, a species suitable for BSL3 studies. While native to Asia, these squirrels are kept as pets and can be examined under controlled conditions.

Intranasal inoculation with Clade IIb IMB 2022 resulted in minimal viral DNA detection in oral swabs, suggesting limited viral shedding. Only one-third of the squirrels seroconverted, indicating low viral replication and immune response. While this MPXV strain appears to have limited virulence in this model, the results may not be directly applicable to other squirrel species. Also, viruses from different clades may be more virulent in Swinhoe's squirrels. Additionally, studies in Mastomys rodents have shown that genital inoculation leads to higher transmission than with respiratory inoculation, emphasizing the importance of studying different routes of infection [13].

The CAST/EiJ mouse model remains a valuable tool for analyzing orthopoxvirus genetics and virulence [12]. While these mice exhibit high susceptibility to MPXV due to aberrations in innate immunity, they remain immunocompetent and mount protective adaptive immune responses [12]. Consistent with previous studies, Clade IIa demonstrated greater virulence than Clade IIb, supporting the hypothesis that MPXV may be adapting to human hosts at the cost of fitness in other species [14].

MPXV evolution involves gene loss, recombination, and genomic rearrangements. The RKI335 strain, derived from Clade IIb B1, displayed unique structural modifications and increased transmission potential due to sound replication in the upper respiratory tract, as confirmed by viral DNA detection in nasal conchae samples. Notably, Variola virus, the orthopoxvirus most adapted to humans, exhibited minimal morbidity in CAST mice, further highlighting the possibility that MPXV is undergoing adaptive changes favouring human-to-human transmission [15].

Although mouse models have limitations in predicting human virulence, CAST mice remain useful for characterizing different MPXV isolates and assessing the role of gene gain and loss in viral adaptation. Future research should focus on evaluating additional host species and infection pathways to refine our understanding of MPXV evolution and transmission risks.

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