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Letter

Thermal tolerance and inactivation of Ebola virus

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Dear Editor,

Viruses of the genus *Orthoebolavirus* cause sporadic outbreaks of severe haemorrhagic fever, with case fatality rates ranging from 25% to 90% (Mahanty and Bray, 2004). Six species of the virus (*Orthoebolavirus zairense*, *sudanense*, *bundibugyoense*, *taiense*, *restonense*, and *bombaliense*) have so far been identified (Biedenkopf et al., 2023). Among these, *Orthoebolavirus zairense*, commonly known as Ebola virus (EBOV), stands out as the most virulent. Given its high contagiousness and lethality, EBOV must be manipulated under biosafety level 4 (BSL-4) conditions, as stipulated by the the People's Republic of China's list of human pathogenic microorganisms (National Health Commission of the People's Republic of China, 2023). Prior to being removed from a BSL-4 laboratory, it is imperative that infectious EBOV undergoes complete inactivation. Here we systematically evaluate viral thermostability under BSL-4 containment conditions, demonstrating EBOV's marked thermotolerance.

The EBOV stock (Mayinga, 1976 strain) was dispensed into 0.2 mL aliquots within 2 mL hermetic screw-cap tubes for thermal challenge experiments. Samples allocated for 4 °C, ambient temperature (22°C–25 °C), and 37 °C treatments were maintained under refrigerated conditions, ambient bench conditions, and an incubator, respectively. Comparative validation studies were performed using both dry-bath and water-bath heating systems at 60 °C and 100 °C. Temperature monitoring was conducted throughout the experiment using a calibrated thermometer in a non-spiked medium sample run concurrently. Post-treatment protocols involved immediate thermal arrest through flash-cooling on ice (15 minutes), followed by refrigerated storage (4 °C) until completion of all experimental conditions. The infectivity of the treated samples was determined using median tissue culture infectious dose ($TCID_{50}$) assay. Briefly, monolayers of Vero E6 cells grown in 96-well plates were inoculated with 100 μL of serial 10-fold diluted samples in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal bovine serum (FBS). Viral titers were calculated by the Reed & Muench method, based on the cytopathic effect (CPE) observed at 10 days post-infection (Cao et al., 2023).

A one-week storage of EBOV at 4 °C resulted in a significant reduction in viral infectivity, dropping from an initial titer of 8.38 $\log_{10}$ $TCID_{50}$/mL to 6.62 $\log_{10}$ $TCID_{50}$/mL. However, the decrease was not marked for storage periods of 1 or 2 days at 4 °C (Fig. 1A). This suggests that EBOV stocks can be safely stored at 4 °C for at least two days without a significant loss in infectivity, thus avoiding the need for repeated freeze-thaw cycles. In contrast, the infectivity of the samples stored at room temperature and 37 °C decreased significantly and progressively over time. After one day, a reduction of approximately 1 $\log_{10}$ $TCID_{50}$/mL in viral titer was observed, with further reductions of 1.6 $\log_{10}$ $TCID_{50}$/mL at room temperature and 2 $\log_{10}$ $TCID_{50}$/mL at 37 °C after two days. Notably, viral infectivity became undetectable after a seven-day incubation period (Fig. 1A). Dry-bath treatment at 60 °C reduced viral titers from 8.38 to 3.82 $\log_{10}$ $TCID_{50}$/mL within 15 minutes, yet residual infectivity (2.63 $\log_{10}$ $TCID_{50}$/mL) persisted after 2 hours (Fig. 1B). Comparable water-bath treatment showed similar kinetics, with titers declining from 7.5 to 2.7 $\log_{10}$ $TCID_{50}$/mL in 15 minutes and remaining at 1.7 $\log_{10}$ $TCID_{50}$/mL after 2 hours (Fig. 1B). Control samples maintained at room temperature showed no titer reduction. Critical differences emerged at 100 °C: dry-bath treatment required 10 minutes for complete inactivation, with detectable virus (1.62 $\log_{10}$ $TCID_{50}$/mL) remaining after 5 minutes (Fig. 1C). Water-bath treatment achieved complete inactivation within 5 minutes (Fig. 1C). Three sequential blind passages in Vero E6 cells confirmed complete inactivation, with no cytopathic effect or detectable EBOV RNA in third-passage supernatants (Fig. 1D). Methodological analysis revealed that incomplete tube immersion in dry-bath systems compromised inactivation efficacy. When 30 μL aliquots were deliberately placed in unheated tube regions (extending beyond the heating block), 90% of samples (9/10) retained high infectivity (7 $\log_{10}$ $TCID_{50}$/mL) after 10-min 100 °C treatment (Fig. 1C), demonstrating the critical importance of complete sample immersion for reliable thermal inactivation.

Our study demonstrates the notable thermostability of EBOV. Existing literature presents variable outcomes across experimental conditions. Mitchell et al. (1984) demonstrated effective viral inactivation in serum samples through 60 °C exposure for 60 minutes while

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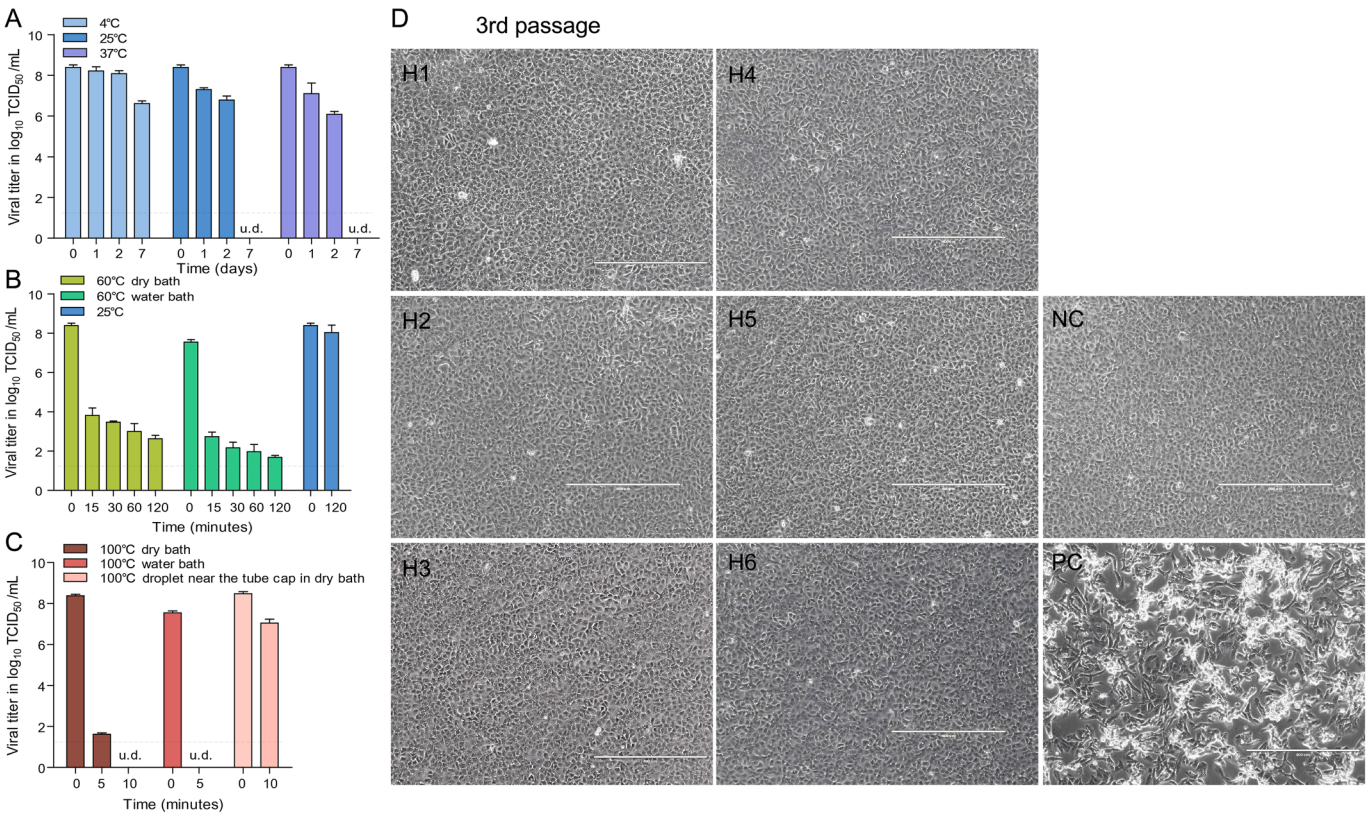

Fig. 1. Thermal treatment of EBOV stock. **A** EBOV aliquots were heat-treated at 4 °C, 25 °C, or 37 °C for indicated durations (n = 3). **B, C** Additional aliquots were treated at 60 °C or 100 °C (n = 3). Specifically, samples near tube caps were incubated at 100 °C in a dry bath for 10 minutes (**C**) (n = 9). Viral infectivity was assessed by $TCID_{50}$ assay, with untreated stock serving as control. The dashed line indicates the assay's detection limit (1.25 $\log_{10}$ $TCID_{50}$/mL); u.d. = undetectable. **D** Replication competence evaluation: EBOV aliquots were either (i) heated at 100 °C (dry bath, 10 min; samples H1–H3) or (ii) boiled at 100 °C (water bath, 5 min; samples H4–H6), then inoculated into Vero E6 cells for blind passage. Untreated virus (PC) and culture medium (NC) served as positive and negative controls, respectively. Third-passage cultures were examined by brightfield microscopy for cytopathic effects (CPE) as evidence of viral infection. Scale bar = 400 μm.

maintaining biochemical stability of glucose, blood urea nitrogen, and electrolytes. Conversely, Smither et al. (2015) documented thermal resistance in murine whole blood specimens, with EBOV-Kikwit remaining infectious following 60 °C exposure for 15 minutes. Haddock et al. (2016) achieved EBOV inactivation by boiling the virus stock at either 100 °C for 10 minutes or 120 °C for 5 minutes, though 65 °C treatment for 30 minutes retained viral infectivity in cellular assays. Olejnik et al. (2023) reported effective inactivation (60 °C/30 min) for samples containing $\leq 1.67 \times 10^6$ infectious particles, while demonstrating infectious residual for samples with higher viral concentrations.

Based on these observations and our experimental validations, we categorically advise against dry-bath methodologies and advocate for complete sample submersion in water-bath systems. Our optimized protocol specifies immersion in boiling water (100 °C) for ≥ 15 minutes. Critical parameters influencing thermal efficacy include: 1) viral strain characteristics, 2) aggregation state, 3) matrix composition, 4) initial viral titer, and 5) thermal transfer dynamics mediated by equipment specifications (Smither et al., 2015; Olejnik et al., 2023; Chung et al., 2007; Negovetich et al., 2010; Tuladhar et al., 2012; Pastorino et al., 2020; Feng et al., 2021). This multifactorial dependence necessitates institution-specific validation of inactivation protocols through rigorous quantitative assays prior to implementation in standard operating procedures. Strict adherence to validated thermal parameters remains imperative for biosafety compliance across all subsequent applications.

FOOTNOTES

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