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Of Mice and Mike—An Underappreciated Ebola Virus Disease Model May Have Paved the Road for Future Filovirology

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Abstract

In 1998, Mike Bray and colleagues published the first immunocompetent laboratory mouse model of Ebola virus disease. Often labeled by peer reviewers as inferior to large nonhuman primate efforts, this model initially laid the foundation for the recent establishment of panel-derived cross-bred and humanized mouse models and a golden hamster model. Nonhuman primate research has always been associated with ethical concerns and is sometimes deemed scientifically questionable due to the necessarily low animal numbers in individual studies. Independent of these concerns, the now-global severe shortage of commercially available large nonhuman primates may pragmatically push research toward increased and improved rodent modeling that may altogether replace nonhuman primate studies in the short term as well as in an optimal future.

Keywords

Ebola; ebolavirus; mouse model; rodent

Mike Bray—our colleague at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) and then at the Integrated Research Facility at Fort Detrick (IRF-Frederick) in Maryland and, until very recently, the longtime editor-in-chief for *Antiviral Research*—made many important contributions to Risk Group 3 and 4 virology. Standing out among them is a publication that may seem trivial to some at first glance: In 1998 (and as a reprint in 1999), Mike and colleagues published the first mouse model for Ebola virus disease (EVD) (Bray et al., 1998, 1999).

In Risk Group 3 and 4 virology (i.e., in research involving highly lethal pathogens such as Ebola virus [EBOV]), animal modeling remains a necessary and often mandatory part of medical countermeasure (MCM) development and efficacy evaluation in the U.S. and a critical part of basic research of infection pathogenesis (Korch et al., 2011). If performed

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correctly, the development of a useful animal model for a particular viral infection and/or disease is a complex and often-challenging endeavor that involves extensive planning.

Animal studies begin with rather mundane but highly critical inquiries into animal availability (types and quantity), individual animal cost, and presence of infrastructure and trained staff to appropriately handle them safely and humanely. Ethical concerns about the use of animals add a layer of continuously changing complexity, because there is no public consensus on whether and how animal experiments should be performed and which types of animals ought not be used. (For example, arthropod research typically incites much less opposition from the public than nonhuman primate research.)

Next, animal experiments need to achieve internal validity (i.e., the experimental design should be able to yield results that are reliably reproducible, ideally in a blinded, randomized, and tightly controlled fashion). In maximum-containment settings (i.e., in the U.S., in animal biosafety level 4 [ABSL-4] facilities tailored to safely and securely work with Risk Group 4 pathogens), compromises are often made despite their potential impact on internal validity (Geisbert et al., 2015); in particular, in many facilities, it is argued to be logistically difficult to achieve blinding of studies, to apply objective euthanasia criteria, and to perform study iterations to increase confidence in reproducibility. In addition, the number of animals used for a study—critical to achieve statistical significance of results—is frequently inversely related to the size and cost of the selected type of animal. Thus, study designs face a paradox of power, i.e., that the least reliable results (even if statistically significant) are achieved for the most suitable animals, and vice versa.

Finally, the experimenter is left with the most important question: *was what was intended to be modeled actually modeled*? Getting to this answer requires consideration of the predictive, face, and construct validities of the model (together referred to as “external validity”). As originally outlined by Willner in 1984, predictive validity measures how well a model predicts a future outcome (e.g., efficacy of a therapeutic); face validity measures how well a model replicates the disease presentation in humans; and construct validity measures how well the experimental mechanics used to model the human phenotype actually reflect the human situation (e.g., route of exposure). In general, predictive validity typically holds the most weight (Willner, 1984), although, in Risk Group 4 pathogen research, face validity is typically emphasized. Because nonhuman animals are not humans, no animal model of a human disease can score absolute validity in all three categories. Accepted as logical, the consequence of this deduction is often glossed over, namely that any animal model of an infectious disease is imperfect in some way or, vice versa, *that any animal model of an infectious disease may be perfect for one particular question*. So, it follows that a combination of highly diverse animal models that complement each other is the best approach to achieve overall validity; one model should not be dismissed for another without carefully examining the scientific question to be addressed.

EBOV, the most notorious member of mononegaviral family *Filoviridae*, causes the frequently lethal EVD in humans that, until very recently, could not be treated beyond general supportive measures (Jacob et al., 2020). The absence of specific MCMs, the high case fatality rate (≈44%), and concerns about nefarious use as biological weapons has

confined, and still confines, animal research with replicative EBOV to ABSL-4 facilities. The U.S. Food and Drug Administration's "Animal Rule" has been interpreted to mean that animal models are mandatory for MCM licensure (Korch et al., 2011). Hence, EVD MCM development and efficacy testing was and still is performed in animal models of EVD. Until 1998, there were only a few that were reasonably standardized upon EBOV exposure: domesticated guinea pigs (*Cavia porcellus* (Linnaeus, 1758)) and large nonhuman primates, i.e., crab-eating macaques (*Macaca fascicularis* Raffles, 1821), hamadryas baboons (*Papio hamadryas* (Linnaeus, 1758)), grivets (*Chlorocebus aethiop*s (Linnaeus, 1758)), and rhesus monkeys (*Macaca mulatta* (Zimmermann, 1780)) (Kuhn, 2008). These models were repeatedly demonstrated to be uniformly lethal after EBOV exposure via different routes and thereby deemed *practical* for MCM evaluation in requiring relatively low animal numbers to achieve statistical significance of outcomes ("stringent" models). As we know today, these models have shortcomings in almost all aspects of external validity, including most importantly in accurately mimicking the natural history of human infection and disease. For instance, rhesus monkeys and crab-eating macaques—long considered by some the "gold standards" for EVD modeling—almost uniformly succumb after EBOV exposure; however, even without specific countermeasures, approximately 50% of EVD patients survive and subclinical infections have been described as part of a much more heterogeneous spectrum of infection and disease. Moreover, the onset (much earlier) and pace of disease (evolving at least twice as rapidly) is also poorly modeled when compared to human disease dynamics. In addition, absent "natural" survival, the individual and public-health consequences of clinical sequelae and EBOV persistence cannot currently be modeled (Jacob et al., 2020; Kuhn et al., 2020) beyond individual case studies (Worwa et al., 2022) or retrospective analyses of tissues archived from individual survivor animals (Zeng et al., 2017). All taken, the face validity of the macaque models of EVD is perhaps not as robust as often stated.

Current large nonhuman primate models are also compromised in construct validity: EBOV exposure via typical intramuscular injection or small-particle aerosol clearly does not mimic the mucocutaneous exposure that is dominantly characteristic of human-to-human transmission (Jacob et al., 2020; Kuhn, 2008; Kuhn et al., 2020). Predictive validity has not yet been attempted systematically in any EVD animal model; indeed, dis-proofs of principle have been described. For example, remdesivir, a small nucleotide antiviral that could protect macaques after exposure by multiple routes, failed in human clinical trials relative to therapeutics *that had looked equally promising in a macaque model* (Mulangu et al., 2019). Clearly, additional animal models are needed to complement or replace the established ones.

Channeling Mike's passion for literature (his impressive personal library is unforgettable), we wonder whether he ever raised an eyebrow at the superficial resemblance of the perception of his paper (Bray et al., 1998) to John Steinbeck's famous novella *Of Mice and Men*, the title of which has become an oft-misused trope in the scientific literature. In this classic, two migrant ranch workers move, in rather senseless iteration, from place to place in search of job opportunities (Steinbeck, 1937). Despite seemingly parallel origin stories, the similarities between the novella and Mike's work begin and end here. In contrast to Steinbeck's lead characters, and even more in contrast to several researchers who dismissed his studies as uncreative busy work, Mike carefully and intelligently planned and performed

the iterative passage of wild-type EBOV in laboratory mice in search of a mouse-adapted strain. As it was already known that immunocompetent adult laboratory mice cannot be infected with wild-type EBOV but newborn mice are susceptible, Mike repeatedly passaged EBOV in progressively older sucking BALB/c mice. Also in contrast to Steinbeck's characters, Mike was successful in his quest: At the end of numerous experiments, he obtained an EBOV strain that, upon intraperitoneal injection of 1–100 plaque-forming units, caused almost uniformly lethal infection in inbred (BALB/c and C57BL/6) and outbred (ICR) strains of adult (5–16-week old) laboratory mice (Bray et al., 1998). Achieving this was a true accomplishment, as evidenced by the fact that it has yet to be repeated for other ebolaviruses, illustrating that even iteration is not trivial.

Though Mike's success was the beginning of numerous studies of mouse-adapted EBOV (MA-EBOV) infection of laboratory mice by many research groups, in particular pertaining T-cell-mediated immunity questions that could not (or cannot) be pursued in domesticated guinea pigs due to the absence of species-specific reagents. However, mouse work carried a stigma. As grant funders and peer reviewers almost inevitably asked for nonhuman primate experiments to confirm results, often invoking the first part of David B. Weiner's famous phrase "mice lie…", they conveniently ignored his follow-up "…and monkeys exaggerate". Thus, the EVD mouse model soon had the reputation of being an inferior model despite the purpose of "the" model not having been defined, and nonhuman primate models were regarded as superior despite the obvious shortcomings. The domesticated guinea pig model was often a compromise, because this model had better face and construct validities compared to laboratory mice. (For general reviews on small animal models in filovirus research see (Banadyga et al., 2018; Bradfute et al., 2012; Yamaoka et al., 2017)).

Briefly reviewed, this premature assessment of the mouse model as inferior is understandable: MA-EBOV-infected mice do not develop some signs of EVD (maculopapular rash, prolonged coagulation times, disseminated intravascular coagulation, and death from shock) present in infected macaques and humans (reduced face validity); infection of mice is only successful via intraperitoneal injection, whereas infection of macaques succeeds by various non-natural routes (even less construct validity); and no MCM that was only tested or succeeded in mice but failed in macaques ever entered the clinic, whereas some antibody-based therapeutic and vaccine candidates that were highly efficacious in macaques also were successful in humans (reduced predictive validity) (Kuhn et al., 2020; Mulangu et al., 2019). In addition, the fact that EBOV had to be adapted through passaging to become pathogenic for laboratory mice or domesticated guinea pigs (GPA-EBOV) has been a never-truly-explained thorn in the sides of nonhuman primate research proponents. It is assumed that MA-EBOV and GPA-EBOV were unjustifiably considered to be somewhat different pathogens than wild-type EBOV despite the very few mutations that were needed to create the adapted viruses (at maximum 13 in the case of MA-EBOV over a ≈19-kb genome) (Banadyga et al., 2016; Ebihara et al., 2006). In the only macaque experiment published, MA-EBOV caused severe disease, as one out of three infected rhesus monkeys succumbed (Bray et al., 2001) (arguably a better representation of EVD case-fatality than the uniformly lethal macaque model with wild-type EBOV). It is not known whether MA-EBOV is pathogenic for humans. However, the report of the death of a laboratory worker who accidentally got infected with GPA-EBOV hints that rodent

adaptation may not fundamentally alter the “character” of EBOV (Stone, 2004; Акинфеева et al., 2005).

Nevertheless, the evident mouse/nonhuman primate model differential clouded the vision of many researchers, who did not recognize the doors opened by Mike’s experimentation. Several now-obvious entries of note have emerged. First, the EVD mouse model enabled the establishment of similar models for other EBOV variants (Chan et al., 2019) and the more distantly related marburgviruses (Warfield et al., 2007; Warfield et al., 2009), suggesting that mouse models could be developed for filovirus disease in general. The established mouse models enabled initial screening and selection of candidate MCMs, including those that ultimately were successfully evaluated in nonhuman primates and then in humans. Second, MA-EBOV enabled the development of a golden hamster EVD model, which has improved face validity compared to the mouse model (Ebihara et al., 2013). Third, and most significantly, the EVD mouse model set the stage for truly *developing* novel immunocompetent animal models tailored to individual needs and specific questions (rather than just using available animals) and, via very limited trial and error, identifying those that develop disease that looks like EVD.

An important step toward tailor-made EVD models was the evaluation and description of outcomes from exposure of Collaborative Cross (CC) multiparental recombinant inbred mice to Mike’s laboratory MA-EBOV (Rasmussen et al., 2014). These CC strains can be used for the analysis of EBOV exposure outcomes (phenotypes) caused by combinatorial allele effects (genome-wide genetic variation) in a randomized large, heterogeneous, and reproducible mouse population (Threadgill et al., 2011). In essence, evaluation of the CC micen may answer the very fundamental modeling question: *What if the wrong mouse strain was used*? Almost all infectious-disease mouse models, including Mike’s, use strains derived from inbred laboratory mice that can be traced back to a founder pair (only two parental genomes) with genetic “contributions” from two to four distinct mice assigned to different *Mus* taxa that were bred using protocols that fundamentally altered their genetics; i.e., these mice are *not* natural/wild-type mice and hence also are not assigned to a species (International Committee on Standardized Genetic Nomenclature for Mice, 2022; Weinstein and Ciszek, 2002). While the various strains derived from this pair differ genetically and phenotypically, due to breeding-derived genetic mosaicism, overall genetic variability is limited. The CC strain was established using eight, rather than two, founder strains of inbred laboratory mice, followed by randomized breeding to create hundreds of independent, increasingly deeply characterized laboratory mouse strains (Collaborative Cross Consortium, 2012; Kollmus et al., 2020).

Sure enough, intraperitoneal exposure of distinct CC strains with Mike’s laboratory MA-EBOV resulted in a wide spectrum of mouse strain-dependent phenotypes bound by extremes—i.e., a strain with low to no lethality and another that developed lethal disease with much-improved face validity (e.g., prolonged coagulation times) (Rasmussen et al., 2014). Thus, the CC strains can now be used as a systems biology platform to assess the interaction of genetic diversity and host antiviral responses (Bowen et al., 2016) in the development of disease, which might lead to predictive validity of the model. For instance, transcriptomic profiling of infected CC strains has already enabled EVD outcome prediction

with 75% accuracy (Price et al., 2020). However, the entire CC effort thus far remains dependent on a MA-EBOV strain; exposure of any CC mouse to wild-type EBOV did not result in robust infection, let alone disease (Rasmussen et al., 2014). Thus, a second avenue toward improved models was opened via the development of humanized (hu) mice, with first successes reported in 2015 (Lüdtke et al., 2015). For instance, severely immunodeficient NSG and TKO laboratory mice with human fetal liver and thymus fragments implanted under the renal capsule (hu-BLT mice) developed EVD-like disease even after exposure to isolates of wild-type EBOV variants (Bird et al., 2016; Lavender et al., 2018); hu-NSG-SGM3 and hu-NSG-A2 laboratory mice developed markedly different disease outcomes when exposed to wild-type EBOV or the related (wild-type) Reston virus (REBOV), which is considered nonpathogenic for humans. In contrast, this difference is not apparent in nonhuman primates, which succumb to infections with either virus (Escudero-Pérez et al., 2019; Spengler et al., 2017). The hu-NSG-A2 mice were also used to successfully model diseases caused by EBOV's pathogenic relatives, Bundibugyo virus (BDBV), Sudan virus (SUDV), and Taï Forest virus (TAFV), for which no mouse models had been available (Escudero-Pérez et al., 2019). Most recently, avatar mice transplanted with human donor-specific antigen-presenting cells and T cells were shown to succumb after wild-type EBOV exposure if the transplanted cells originated from EBOV-naïve individuals, but they survived if the cells originated from EVD survivors (Rottstegge et al., 2022).

These efforts are still in the infancy and are burdened with numerous hurdles that must be overcome: Evaluation of all CC strains to identify ideal candidates for different EBOV research questions would be a gigantic endeavor, and any identified candidate would have to be bred and made available. Humanized mice need to be created individually in a time- and cost-intensive manner to potentially preclude experiments with large numbers, essentially negating one of the biggest advantage of "regular" mouse models. Further, the interactions of human (engraft) and murine tissues in human mice (including possible graft-versus-host disease) and how they possibly influence pathogenesis have yet to explored.

However, in their entirety, the varied mouse models have created an overall toolbox that will likely never be available for domesticated guinea pigs or newer EVD models, such as domestic ferrets (Schiffman et al., 2022), let alone for large nonhuman primates. By now, laboratory mice have been established in all colors and shapes (including knock-outs, knock-ins, and other transgenics), and routine tools exist to establish new transgenic mice relatively swiftly. They are commercially available as inbred and outbred lineages, thereby enabling tightly controlled experiments in near-clonal backgrounds or delving into outbred questions of disease heterogeneity. Compared to large nonhuman primates, even the costliest mice are cheap. Most importantly, mice of one type can be cross-bred with another type, thereby enabling, for instance, the creation of particular transgenic CC mice; and, of course, CC mice could also be humanized. Laboratory mice are likely also the best genetically and immunologically characterized mammal, enabling systems biology approaches at resolutions that are unlikely to be achieved in other animals. The combination of all these opportunities with, for instance, mouse-specific mouse/human-specific organ-on-chip or organoid technologies should ultimately enable the creation of "made-for" EBOV infection or EVD models that could be used to answer almost any question in ways that likely will remain impossible for any non-mouse model. Therefore, the current severe global

shortage of large nonhuman primates that developed as a result of the COVID-19 pandemic is not a setback for EBOV research; rather, it is a golden opportunity to refocus and develop improved models that will produce more reliable results in less ethically controversially settings.

Influential researchers are often stated to be “standing on the shoulders of giants” (Isaac Newton, 1675). Mice of all colors and shapes, perhaps holding keys to important downstream doors, sit on Mike Bray’s broad shoulders; *of mice and a man*, indeed.

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