

Considerations in the Use of Nonhuman Primate Models of Ebola Virus and Marburg Virus Infection

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The filoviruses, Ebola virus and Marburg virus, are zoonotic pathogens that cause severe hemorrhagic fever in humans and nonhuman primates (NHPs), with case-fatality rates ranging from 23% to 90%. The current outbreak of Ebola virus infection in West Africa, with >26 000 cases, demonstrates the long-underestimated public health danger that filoviruses pose as natural human pathogens. Currently, there are no vaccines or treatments licensed for human use. Licensure of any medical countermeasure may require demonstration of efficacy in the gold standard cynomolgus or rhesus macaque models of filovirus infection. Substantial progress has been made over the last decade in characterizing the filovirus NHP models. However, there is considerable debate over a variety of experimental conditions, including differences among filovirus isolates used, routes and doses of exposure, and euthanasia criteria, all of which may contribute to variability of results among different laboratories. As an example of the importance of understanding these differences, recent data with Ebola virus shows that an addition of a single uridine residue in the glycoprotein gene at the editing site attenuates the virus. Here, we draw on decades of experience working with filovirus-infected NHPs to provide a perspective on the importance of various experimental conditions.

Keywords. Ebola virus; Marburg virus; filovirus; nonhuman primate; animal model; vaccine; treatment.

Mice, hamsters, and guinea pigs have all been developed as animal models of infection for a number of species and/or strains of Ebola virus (EBOV) and Marburg virus (MARV) [1–6]. There are a number of features of disease that rodents have in common with humans and nonhuman primates (NHPs), and mice, hamsters, and guinea pigs have all served well as early screens for evaluating candidate vaccines and antiviral drugs. However, there are significant differences between these disease models. Most notably, filovirus isolates derived from primates do not typically produce disease in

rodents upon initial exposure. Serial adaptation is needed to produce a uniformly lethal infection in immunocompetent rodents. Also, blood coagulation abnormalities, which are an important feature of filovirus hemorrhagic fever in primates, are not as prominent in older rodent models [7, 8]. There is relatively little evidence of disseminated intravascular coagulation (DIC) in mouse or inbred strain 13 guinea pig models of EBOV or MARV infection. The more recently developed hamster and outbred Hartley strain guinea pig models of filovirus infection do show coagulopathy that is more consistent with disease in primates [5, 9, 10]. Given their better clinical parallels to human disease, we suggest that these improved rodent models should be used to assess the potential of any candidate countermeasure prior to evaluation in NHPs.

Published studies have indicated that filovirus infection in NHPs faithfully reproduces what is known about human disease [11, 12]. The incubation period appears to be similar to human responses but largely depends

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on the route and dose (discussed below). Initial signs and symptoms are characterized by an abrupt onset with flu-like signs, including fever, malaise, and myalgia, followed by anorexia, lethargy, nausea, vomiting, and diarrhea. Hemorrhagic manifestations can develop, particularly in severe cases at the peak of illness, and include petechiae, uncontrolled bleeding from venipuncture sites, epistaxis, and other mucosal hemorrhages. Fatal cases manifest with hypovolemic shock and multiple organ failure. Hematologic features include lymphopenia, neutrophilia, and thrombocytopenia. Liver and lymphoid tissues (spleen and lymph nodes) are important organs in the disease pathogenesis. Elevated levels of liver-associated enzymes, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as blood coagulations biomarkers, including D-dimers, are prominent in filovirus infection of primates. Monocytes, macrophages, and dendritic cells are primary sites of filovirus replication in primates, and it is thought that high levels of tissue factor expression by filovirus-infected monocytes and macrophages trigger DIC. A dysregulated proinflammatory cytokine/chemokine response is also thought to be an important feature of filovirus infection in humans and NHPs. As nearly all aspects of filovirus infection in humans recapitulate in macaques and other species, NHPs are the most valuable animal model of human disease. Here, we provide perspectives on a variety of topics related to filovirus NHP studies, ranging from an overview on the use of NHPs for filovirus research to our own perspectives on the use of different experimental conditions.

NHP SPECIES FOR MODELING EBOLA VIRUS AND MARBURG VIRUS HEMORRHAGIC FEVER

Several NHP species have been used as models of EBOV and/or MARV infection, including African green monkeys (*Chlorocebus aethiops*), cynomolgus macaques (*Macaca fascicularis*), rhesus macaques (*Macaca mulatta*), marmosets (*Callithrix jacchus*), and hamadryad baboons (*Papio hamadryas*) [11, 12]. African green monkeys [13, 14] and marmosets [15] do not present with the maculopapular rash that is a feature of disease in humans, macaques, and baboons, casting doubt on their accuracy as models of human filovirus infection. There have been few studies using baboons to model filovirus infection. Baboons also present logistical challenges in biosafety level 4 (BSL-4) facilities, given their large size, space constraints, and safety concerns. Cynomolgus and rhesus macaques have been the most widely used NHP species for studying filovirus infections and for testing candidate vaccines and treatments. Cynomolgus macaques have been the species most often used for vaccine studies, while rhesus macaques have been more frequently used for evaluating postexposure treatments. This is most likely because, all conditions being equal, the disease course appears on average slightly faster in cynomolgus macaques than rhesus macaques

[11]. Nonetheless, the features of disease in both cynomolgus and rhesus macaques appears to best reproduce the human condition among the NHP species.

SPECIES AND STRAINS OF FILOVIRUSES

The pathogenicity of filoviruses in humans is highly variable and primarily depends on the species or strain. This phenomenon is recapitulated in NHPs. Among the 5 species of EBOV, *Zaire ebolavirus* (ZEBOV) causes the most-rapid and most-severe disease in macaques. Experimental infection of macaques with 1000 plaque-forming units (PFU) of ZEBOV by various routes of exposure typically results in uniform lethality regardless of the strain of ZEBOV. The passage history of the seed stocks in cell culture appears to influence the disease course, with stocks that have been passaged fewer times producing a more rapid disease course. This is most notable with the 1995 Kikwit strain where it has been shown that increased passaging of the virus results in the incorporation of an additional uridine (U) residue in the glycoprotein (GP) gene editing site [16, 17]. Normally, GP is expressed through a transcriptional editing event that leads to the insertion of an extra U residue at the editing site. This mutation has biological consequences, as a virus with an editing site of 7 U residues mainly produces a secreted, nonstructural GP (sGP), whereas a virus with an 8-U residue editing site mainly produces GP, which forms the virion surface spike structures [18–20]. So seed stocks with a high proportion of virus populations with 8 U residues at the GP editing site will not produce as much sGP as wild-type seed stocks primarily having a 7-U sequence. The function of sGP is not completely known, but it is thought to be involved in antigenic subversion to evade host immunity [21]. Importantly, stocks of ZEBOV Kikwit strain that have higher proportions of 8 U residues at the GP editing site while producing near uniform lethality in NHPs have been associated with a slower disease course in macaques than seed stocks with low proportions of 8 U residues at the GP editing site [22] (T. W. Geisbert, unpublished observations). This is critical when assessing and triaging patients for determining medical countermeasures, as even slight delays in the course of disease can result in survival or nonsurvival. As an example, an adenovirus serotype 5–based ZEBOV GP vaccine that was previously shown to completely protect cynomolgus monkeys against a ZEBOV Kikwit seed stock containing a low proportion (approximately 34%) of 7 U residues at the GP editing site only partially protected cynomolgus monkeys under near identical test conditions when challenged with a ZEBOV Kikwit seed stock in which the content of the 7-U phenotype was >99% [23].

Fewer NHP studies have been done with non-ZEBOV species of EBOV [11, 12]. The disease course for *Sudan ebolavirus* (SEBOV) appears to be slower in cynomolgus and rhesus macaques than ZEBOV with all other conditions being equal, and

there have been more occurrences of surviving animals. Studies in cynomolgus macaques exposed to 1000 PFU of virus also show a slower disease course for *Bundibugyo ebolavirus* (BEBOV) and *Ivory Coast ebolavirus* (ICEBOV), with mortality rates of 60%–75%. While *Reston ebolavirus* (REBOV) does not appear to be associated with lethal disease in humans, it causes 80%–100% mortality in cynomolgus monkeys, with a disease course that appears to progress similar to or slightly slower than disease due to BEBOV and ICEBOV.

When comparing medical countermeasures, it is advisable to use the most virulent species or strains of filoviruses because successful interventions against a more aggressive strain can likely be adapted easily to less virulent strains. In addition, consideration should be given to historical data, particularly if historical controls are being used for particular seed stocks. For ZEBOV, the large majority of NHP studies have used the Kikwit strain. It is an open question whether future studies should use the West African outbreak strain (Makona) or whether studies should continue to use Kikwit strain seed stocks that contain high populations of 7 U. Arguments could be made for both choices, but the scientific information gleaned from either could and has been equally revealing. For other species of EBOV, there is even less clarity. NHP studies with SEBOV have used both the 1976 Boniface strain and the 2000 Gulu strain. In most cases, the passage level of the Gulu strain is lower, and many laboratories have opted to use this strain. For BEBOV and ICEBOV, there are few strains available, and all work in NHPs for either species has been done with single strains. For MARV, there is a clear difference in virulence among strains, with the Angola strain producing a much more rapid and severe disease [10, 24, 25], and any assessment of medical countermeasures against MARV will benefit from using the more virulent Angola strain following the same rationale as mentioned above for EBOV.

CHALLENGE DOSE AND ROUTE

While the most accurate and consistent method for quantifying filoviruses is debatable, PFU has been used to measure the level of infectious filovirus particles in nearly all NHP studies. Experimental studies in NHPs have shown that doses ranging as low as 2–15 PFU, administered by a variety of challenge routes, can produce a lethal filovirus infection [14, 26–28]. It is likely that the dose of exposure causing infection during a natural outbreak or that would be encountered in the event of deliberate misuse varies widely. The most important consideration in regard to the route and dose of challenge virus used is how the disease observed in NHPs compares human disease. Any combination of route and dose should faithfully reproduce the human condition as accurately as possible. For financial, logistical, and ethical reasons, NHP studies are often conducted using small numbers of animals, compared with studies using

rodents. To achieve statistical significance, challenge doses high enough to produce uniform lethality are desirable. However, for all filoviruses, infection is not uniformly lethal under natural conditions.

The course of disease appears to be influenced by the dose of filovirus used. As an example, cynomolgus macaques exposed by intramuscular injection with a low challenge dose of ZEBOV (approximately 10 PFU) died from infection 8–12 days after challenge [27], but those exposed to a high dose (1000 PFU) died 5–8 days after challenge [11, 12]. Likewise, a similar protraction of disease course in NHPs concurrent with serial dilution was noted for MARV [11]. In human cases, route of infection ostensibly affects the disease course and the outcome. The mean incubation period for cases of ZEBOV known to be due to injection was 6.3 days, compared with 9.5 days for contact exposures [29]. Moreover, the case-fatality rate in the original 1976 ZEBOV outbreak was 100% (85 of 85) in cases associated with injection compared with 80% (119 of 149) in cases of known contact exposure [29]. Although the NHP models appear to be exquisitely sensitive to the filoviruses, compared with humans, particularly cynomolgus macaques for ZEBOV, this observation in part could be attributed to the fact that most NHP studies involve intramuscular injection with very high challenge doses.

While filoviruses have been shown to produce lethal disease in NHPs when challenged by a variety of doses and routes, including aerosol [14, 28], oral [30], and conjunctival [30], the most frequently used challenge route and dose in filovirus NHP studies has been 1000 PFU by intramuscular injection [11, 12]. While the 1000 PFU dose for the intramuscular challenge may appear high to model contact exposures, it is not high relative to exposures entering directly through a break of the skin. Thus, it is likely that direct injection of the challenge virus into the muscle of NHPs better mimics human cases of infection through a needle stick or break in the skin. This test condition was originally established and adopted by most BSL-4 facilities because it represents a likely scenario of an accidental needle stick involving either medical staff performing procedures on an infected patient or laboratory staff performing procedures on an infected animal. Given that, at peak stages of disease, infected humans and NHPs can have viremia levels on the order of 10^6 – 10^8 PFU/mL, it is not hard to envision an exposure from a needle stick being at least 1000 PFU. If an overall goal of combating filovirus infection is to develop vaccines or treatments to protect against all types of exposures, not just a proportion of cases that may mostly result from contact exposures to mucosal surfaces, then the conditions being used at 1000 PFU (more stringent) are best. This is also important in terms of triaging medical countermeasures so that the limited funds available for advanced development of vaccines and treatments are allocated to the most promising candidates.

To the best of our knowledge, the 1000 PFU challenge dose and intramuscular route have produced 100% lethality in

cynomolgus macaques challenged with either 7-U or 8-U virus stocks of ZEBOV. The situation with rhesus macaques is slightly different, such that these conditions have not produced 100% lethality for ZEBOV, with each facility having rare surviving control rhesus macaques (Table 1). However, it appears that the surviving rhesus macaques were associated mainly with challenge by the 8-U ZEBOV seeds stocks and not 7-U ZEBOV seed stocks. In addition, a lower challenge dose of <700 PFU of ZEBOV in the rhesus macaque model may result in more surviving control animals [31] (Table 1).

Overall, in regard to challenge route, to achieve adequate statistical power by using realistic numbers of NHPs it is essential that models are uniformly or near uniformly lethal as possible. In most cases, this means using challenge doses of at least 1000 PFU. This is achievable for MARV, ZEBOV, and, in most cases, SEBOV, depending on the species of macaque used. For BEBOV and ICEBOV, this will be more problematic because uniform lethality or near uniform lethality has not been observed in any species of NHP [11, 12].

EUTHANASIA CRITERIA

Perhaps the most controversial topic in the use of NHP models for filovirus infection is how to define criteria for euthanasia. For studies in which survival is the primary end point, variability in results among different BSL-4 laboratories may be caused by differences in determining when to euthanize an animal. While consistency in criteria among laboratories is a worthy

goal, standardization of criteria among facilities is difficult for a variety of reasons. From a regulatory perspective, animal work at each institution is overseen by independent institutional animal care and use committees (IACUCs). Each IACUC ultimately approves criteria used for determining when euthanasia is required. Because these committees are transient by nature, criteria can change from time to time even within the same institution. Having IACUCs from multiple institutions all agree on the same exact euthanasia criteria seems impossible. In addition, while IACUCs follow the same regulatory guidelines in the United States, there is inherent subjectivity in some euthanasia criteria.

There have been several attempts to identify biological parameters or biomarkers that indicate before death that an animal will or will not survive. However, this has proven to be quite difficult, and no such biomarker or algorithm exists to date. In particular, because there are pathological differences associated with the various species and strains of filoviruses, conditions would need to be established independently for each filovirus seed stock and for each species of NHP. NHPs are outbred, and animal-to-animal variability further complicates this goal, particularly given the small numbers of NHPs used in most filovirus studies. Various parameters or combinations of parameters have been proposed, including temperature, macular rash, and biomarkers such as circulating tissue-associated enzymes. However, individually none of these metrics can accurately predict outcome. Fever has no association with survival, nor does the presence of a macular rash or degree of the rash. Viral load

Table 1. Experimental Positive Control Nonhuman Primates (NHPs) Infected With *Zaire ebolavirus* (ZEBOV)

Laboratory	NHP Species	ZEBOV Strain	7 U or 8 U ZEBOV Stock	Challenge Dose	NHPs, No.	Surviving NHPs, No.
NML	Cynomolgus	Kikwit	7 U	800–1200	27	0
NML	Rhesus	Kikwit	7 U	800–1200	11	0
NML	Rhesus	Kikwit	7 U	600–700	6	1
RML	Cynomolgus	Kikwit	8 U	Approximately 1000	9	0
RML	Cynomolgus	Kikwit	7 U	Approximately 1000	3	0
RML	Rhesus	Kikwit	8 U	Approximately 1000	5	2
RML	Cynomolgus	Mayinga	7 U	Approximately 1000	6	0
RML	Rhesus	Mayinga	7 U	Approximately 1000	15	0
RML	Cynomolgus	Makona	7 U	Approximately 1000	6	0
GNL	Cynomolgus	Kikwit	7 U	800–1200	11	0
GNL	Cynomolgus	Kikwit	8 U	800–1200	5	0
GNL	Rhesus	Kikwit	7 U	800–1200	6	0
GNL	Cynomolgus	Makona	7 U	Approximately 1000	2	0
GNL	Rhesus	Makona	7 U	Approximately 1000	5	0
USAMRIID ^a	Cynomolgus	Kikwit	8 U	800–1200	37	0
USAMRIID ^a	Rhesus	Kikwit	8 U	800–1200	25	1

Abbreviations: GNL, Galveston National Laboratory, University of Texas Medical Branch; NML, National Microbiology Laboratory, Public Health Agency of Canada; RML, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health; USAMRIID, US Army Medical Research Institute of Infectious Diseases.

^a Data are from T. W. G.'s laboratory.

in serum or plasma may be the best indicator of outcome but, in the context of BSL-4 facilities, cannot always be performed immediately and can only be assessed on days when animals are anesthetized for sample collection. Furthermore, the act of sedating an already sick animal may hasten their demise, thereby affecting results. Certain circulating tissue-associated biomarkers such as AST, ALT, γ -glutamyl transpeptidase, blood urea nitrogen, and creatinine, among others, which can also only be assessed on days where animals are anesthetized for sample collection, have been suggested as possible indicators. Combinations of these markers have also been proposed as indicators, again with limited usefulness. Furthermore, for each algorithm proposed, there is nearly always an animal or animals that would have met criteria for euthanasia yet ultimately survived. Because NHP studies in BSL-4 facilities most always involve small groups, a mistake in prematurely euthanizing even a single animal can compromise the analysis of the entire study, in which case a repeated experiment unnecessarily uses additional NHPs. For these scenarios, a skilled pathologist is invaluable in assessing each NHP to ensure to the best extent possible that no NHP was prematurely euthanized.

The objectives of each NHP study should be well defined in the experimental design and animal protocol. For some studies survival may be the primary objective, while for other studies the goal may be to prevent severe disease. In the latter case, it may be desirable to implement an early study end point.

There is invariable subjectivity in evaluating animal behavior and any other parameter that cannot be quantitatively measured as euthanasia criteria. Yet the human eye is often the best indicator. There is no substitute for skilled investigators and laboratory staff with extensive experience with filovirus-infected NHPs who monitor the animals closely from times well before exposure of the animals through the study end point. This is particularly important if behavior or activity are used as criteria for euthanasia, because each animal has unique traits and may react differently to different stimuli. In our view, the most important aspect in regard to comparing survival data among different laboratories is the accurate recording of data both in the form of quantitative data, as well as detailed descriptive clinical observations.

REGULATED STUDIES

As a result of the US Food and Drug Administration (FDA) Animal Rule [32], there has been interest as well as confusion regarding the requirement for good laboratory practices (GLP) or “GLP-like” NHP studies in BSL-4 facilities. It is important to note that there are no regulations that specifically address data quality and integrity issues for FDA Animal Rule-specific studies [32]. GLP nonclinical laboratory studies regulations were developed as a quality system for nonclinical safety studies. The FDA does recommend the use of GLP for the few pivotal animal

studies supporting licensure of a vaccine or drug to the extent practicable. However, it is also noted that there may be justifiable limitations in the ability to apply GLP when conducting these studies, specifically for those using challenge agents that require high-containment facilities. Indeed, for all practical purposes, the performance of GLP or GLP-like studies is a near impossibility for most existing BSL-4 facilities as currently constructed. True GLP or even GLP-like conditions require strict separation of studies. Most if not all of the current BSL-4 facilities are constructed as open-space laboratories, hindering separation of individual projects. Most equipment is shared among staff, further complicating the performance of regulated studies. In addition, most BSL-4 animal rooms are constructed to house large numbers of NHPs, meaning that in many cases different studies using the same virus are being conducted in the same room at the same time. Given the very limited amount of BSL-4 facility space for NHPs globally, it is not practical or economically feasible to repeatedly dedicate a single animal room to a single regulated NHP study. The most important aspect of compliance in the spirit of using GLP to the greatest extent practicable may be to ensure the quality and integrity of the data collected. As NHP studies in BSL-4 facilities are inherently time demanding and extremely costly, any attempt to add additional administrative burdens on research staff may only hinder the development of promising countermeasures as a result.

Two of the most effective therapeutics in NHP models are ZMapp and TKM-Ebola [33, 34]. These antivirals have been used to treat repatriated patients in the current West African ZEBOV outbreak [35, 36]. These products, as well as the leading ZEBOV vaccine candidates currently in or soon entering phase 1 trials [37–41], exist because researchers at BSL-4 facilities had the ability to screen and triage very large numbers of candidate countermeasures, something that would not have been possible to this extent if each study had necessitated GLP or GLP-like conditions. Importantly, if ZEBOV continues to emerge in human populations, promising countermeasures will better reach final licensure through advanced clinical development in humans and thus not require licensure under the FDA Animal Rule.

In our view, standardization of conditions for research work is not desirable because it goes against the basic principal of science. Science is competitive, and important findings must be reproducible. Each individual experiment must be properly controlled. Thus, if a study is properly designed and controlled, there is no need to standardize all test conditions. Standardization would limit research in the field to what some researchers decide are the desired conditions to move forward with. This may not reflect real-world challenges, and we may lose the ability to react to naturally occurring problems. Science is based on ethics, experience, and skills. Controlling and dictating to the field on what conditions to use is dangerous and will not pay dividends in the long run. Standardization should only be

applied to a single final pivotal study in NHPs that uses products manufactured under current good manufacturing practices that will be used to support licensure under the FDA Animals Rule. Such conditions are unnecessary for all studies in BSL-4 facilities leading up to a final pivotal study and, as previously mentioned, will only hinder the development of the most promising medical countermeasures.

BLINDED STUDIES

There is no question that blinding of a study eliminates any human bias regarding interpretation or outcome. Blinding may be beneficial or deemed as a requirement for final studies used to support FDA licensure of a medical countermeasure. However, these types of pivotal studies should represent a small percentage of the total number of NHP studies performed in BSL-4 facilities. Because of extensive training requirements to master skills in BSL-4 facilities, most BSL-4 facilities have relatively small teams of staff that perform duties associated with NHP studies. In particular, complex requirements that need to be met for postexposure treatment studies, such as the preparation of clinical modalities, often during off duty hours, to maintain the integrity of the blinding process, increase the burden and therefore limit the number of evaluations that can be performed. Importantly, having staff not associated with the study that could immediately decode the animals in an emergency, such as an occupational exposure, would be needed at all times. While there may be a few cases where blinding of a NHP study has merit, in most cases it has very limited value when weighing costs to benefits in the context of a BSL-4 environment.

CONCLUSIONS

Cynomolgus and rhesus macaques are considered the gold standard animal models for filoviruses. With an increasing number of BSL-4 laboratories working with NHPs, particularly in North America, there is a desire to be able to compare results among the different facilities. Historically, this has been difficult because of different experimental conditions used by each laboratory, including differences in the strains and passage histories of filovirus challenge stocks, routes and doses used to challenge animals, and assays used to measure biomarkers of disease and viral load. Recent efforts by groups such as the Filovirus Animal Nonclinical Group have focused on standardizing experimental conditions for NHP studies among the various BSL-4 facilities. While attempts can be made to minimize differences, such as using the same virus isolates, passage history, challenge routes and doses, species and ages of animals, and standard procedures for various bioassays, it remains difficult and may not be desirable to implement a system whereby all conditions are completely identical. One of the main advantages

of using outbred animals in research is their heterogeneity, a characteristic of the human population. Ensuring that filovirus challenge stocks are exactly the same across facilities is no small task, even if a central facility were able to distribute standard challenge stocks. Given the ever-increasing number of regulations being implemented and required for shipping and storing filoviruses, transportation of infectious material between BSL-4 facilities has become an important challenge. In addition, there are other important conditions, such as plaque assays or scoring criteria for euthanasia among others, that are not easily standardized for which there will always be at least some degree of subjectivity. Genetic differences, even among the same species of NHPs, is also no small condition to attempt to standardize and may not be desirable, again in the interest of generating observations that apply to the human population. The most important aspect of analyzing any medical countermeasure in the NHP models is that survival results be overall reproducible from one facility to another. There have been and will continue to be difference in results among facilities. Identifying the causes and minimizing these differences is a worthy objective. Clearly, in the context of a natural outbreak or intentional release of a filovirus, all conditions are not controlled, and an ideal vaccine or antiviral drug should be efficacious across a range of conditions, and using stringent conditions is likely to relate to broader protective efficacy.

Notes

Disclaimer. The opinions, interpretations, conclusions, and recommendations contained herein are those of the authors and are not necessarily endorsed by the University of Texas Medical Branch at Galveston, the Public Health Agency of Canada, or the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH).

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