

# Physicochemical Inactivation of Lassa, Ebola, and Marburg Viruses and Effect on Clinical Laboratory Analyses

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**Clinical specimens from patients infected with Lassa, Ebola, or Marburg virus may present a serious biohazard to laboratory workers. We have examined the effects of heat, alteration of pH, and gamma radiation on these viruses in human blood and on the electrolytes, enzymes, and coagulation factors measured in laboratory tests that are important in the care of an infected patient. Heating serum at 60°C for 1 h reduced high titers of these viruses to noninfectious levels without altering the serum levels of glucose, blood urea nitrogen, and electrolytes. Dilution of blood in 3% acetic acid, diluent for a leukocyte count, inactivated all of these viruses. All of the methods tested for viral inactivation markedly altered certain serum proteins, making these methods unsuitable for samples that are to be tested for certain enzyme levels and coagulation factors.**

Clinical laboratory support for hospitalized patients suspected of infection with a class 4 viral agent (such as Lassa, Ebola, or Marburg virus) presents a serious biohazard. Fluids, secretions, and wastes from these patients must be considered as infectious and, because of the seriousness of the illness associated with class 4 viral agents, highly dangerous. Samples from these patients should be handled only by experienced laboratory personnel in laboratories equipped with safety facilities of the P-4 level. Unfortunately, only a few such laboratories exist, and they may be limited in the type of clinical analyses that can be performed. For the physician to make important diagnostic and therapeutic decisions, it is vital that the laboratory results be reported without delay. Clearly, the desirable situation is having the capacity to do the necessary tests in the hospital laboratory, with no risk to the laboratory staff.

We have developed a small, portable laboratory unit that is equipped to safely handle these highly infectious samples. In establishing procedures for this portable laboratory, our aim was to inactivate the virus present in a blood specimen without altering the components of the blood which are being assayed. These methods, the results of their application, and their adaptation in a clinical laboratory or to a portable laboratory are the basis for this report.

## MATERIALS AND METHODS

Testing of virus-infected material was performed within the maximum containment laboratory, a P-4 facility, at the Centers for Disease Control.

**Virus strains.** Three virus strains were used in the physicochemical inactivation tests. Known quantities of the individual virus stocks were added to normal human sera or whole blood which had been previously tested and found to be negative for antibodies to each of the three viruses. The Josiah strain of Lassa virus was isolated from human serum in Sierra Leone (2), and a virus stock was prepared from the third passage in Vero cells. Ebola virus strain Mayinga was isolated from human blood in Zaire (8). The virus was passed three times in Vero cells, and a stock was prepared. Marburg virus strain Musokee was isolated from human serum in Kenya (11). It was passed four times in Vero cells, and a

virus stock was prepared. All virus stocks were stored in liquid nitrogen.

**Tissue culture.** A Vero cell line designated as E-6, maintained at the Centers for Disease Control, was used for virus preparation. The cells were grown in Eagle minimum essential medium with the addition of 10% fetal calf serum and the following antibiotics: 1 U of penicillin per ml, 0.5 µg of streptomycin per ml, and 0.02 µg of amphotericin B per ml. Once the cell layer was confluent, the medium was changed, and the fetal calf serum was decreased to 2%.

**Thermal inactivation.** Virus from each stock was added to separate samples of normal human serum samples to give a final virus concentration of  $10^5$  to  $10^7$  PFU/ml of serum. Each serum sample (0.5 ml) was sealed in a plastic vial and totally immersed in a 60°C water bath. Samples of each virus were removed at 10-, 20-, 30-, 40-, 50-, 60-, and 70-min intervals and cooled immediately to ambient temperature (18°C). Each serum specimen was then titrated, and the viral infectivity, expressed in PFU, was determined by virus plaque formation in E-6 cells (12). Survival curves (Fig. 1, 2, and 3), plotting the titer of surviving virus against time at 60°C, were drawn to determine the rate of inactivation. In the equation  $V_s = V_0 + b(\log T)$ ,  $V_s$  is the surviving virus measured in  $\log_{10}$  virus PFU per milliliter after time,  $T$ .  $V_0$  is the initial virus titer in  $\log_{10}$  virus PFU per milliliter,  $b$  is the calculated rate of inactivation ( $\log_{10}$  virus PFU per  $\log_{10}$  minutes), and  $\log T$  is the  $\log_{10}$  of time. The experimental data were also used to plot inactivation curves (Fig. 4) for each virus, showing the time required to inactivate a given titer of virus at 60°C. In the equation  $\log T = \log T_0 - (V_s - V_0)/b$ ,  $\log T_0$  is the  $\log_{10}$  of the time required, from experimental data, to inactivate  $V_0$ .

**Chemical inactivation.** Freshly drawn human blood in potassium EDTA anticoagulant was mixed with each virus stock at a final concentration of  $10^5$  to  $10^6$  virus PFU/ml of blood. A 1:100 dilution of the blood was then made with 3% acetic acid (pH 2.5) and phosphate-buffered saline (pH 7.3) as a control. The dilutions were held for 15 min at room temperature to allow for inactivation of the virus. Each dilution was then assayed for viral infectivity as above.

**Gamma-radiation inactivation.** Samples of human serum and plasma were each divided into two equal portions between 1 and 2 ml. One set of sera and plasma was exposed to a dose of  $1.27 \times 10^6$  rads of gamma radiation. The gamma-radiation source was a gamma cell containing  $^{60}\text{Co}$  (model

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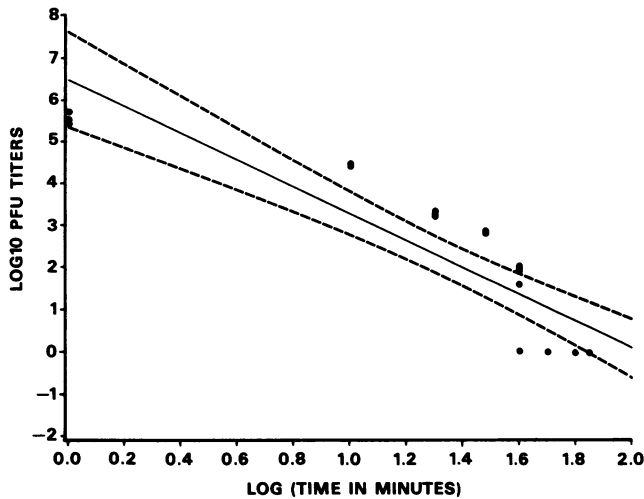


FIG. 1. Inactivation of Lassa virus at 60°C. Symbols: —, regression line; ---, 95% confidence intervals; ●, experimental data points.

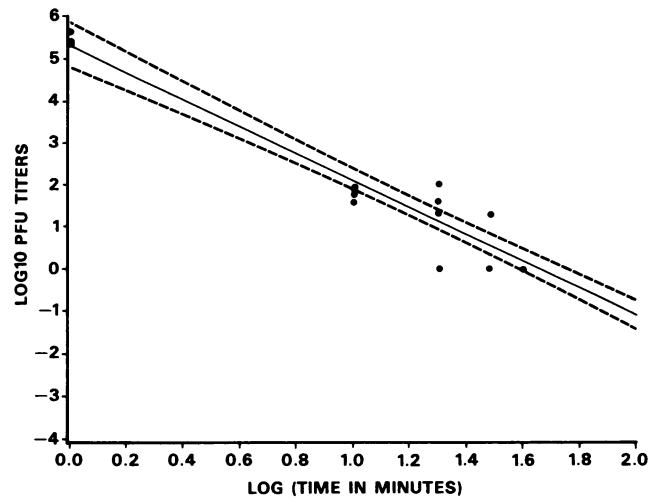


FIG. 3. Inactivation of Marburg virus at 60°C. Symbols: —, regression line; ---, 95% confidence intervals; ●, experimental data points.

220; Atomic Energy of Canada, Ltd., Ottawa, Canada). During the radiation exposure, test samples and controls were kept at 4°C in an ice bath. All samples were then held at 4°C until assayed for serum enzyme levels and coagulation factors.

**Clinical assays.** Serum enzyme levels for aspartate aminotransferase, alanine aminotransferase, creatine kinase, alkaline phosphatase, and gamma-glutamyltransferase were determined for each irradiated and control sample by using the Gilchem reagent system (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Each assay was performed in triplicate. For each of the five enzymes tested, the percentage of enzyme activity lost was calculated for each serum pair. The prothrombin time and the activated partial thromboplastin time were determined photometrically. The percent increase in clotting time was determined for each pair of samples. All tests were performed in duplicate.

To determine the effect of the thermal inactivation method on human serum components, five human serum samples

were each divided into two equal portions of 1 ml each and sealed in test tubes. All tubes labeled A were held at room temperature, and tubes labeled B were immersed in a 60°C water bath for 1 h. The test samples and controls were analyzed on an SMA-6 chemistry analyzer for serum electrolyte, glucose, and blood urea nitrogen levels.

**RESULTS**

In preliminary experiments in which we heated serum at 45, 56, and 60°C for periods of up to 75 min, we found that complete inactivation of the three viruses tested occurred

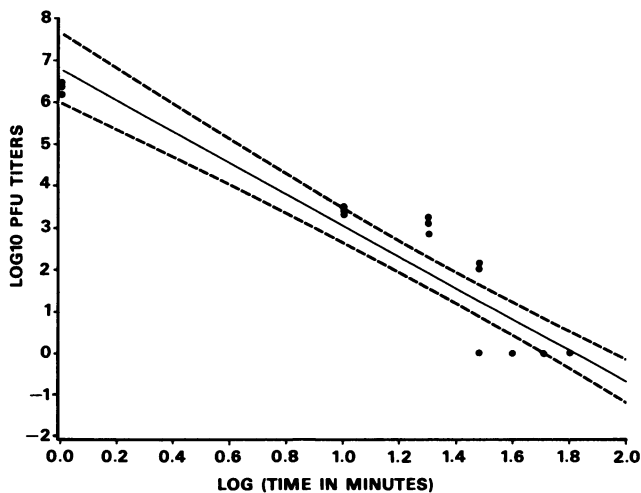


FIG. 2. Inactivation of Ebola virus at 60°C. Symbols: —, regression line; ---, 95% confidence intervals; ●, experimental data points.

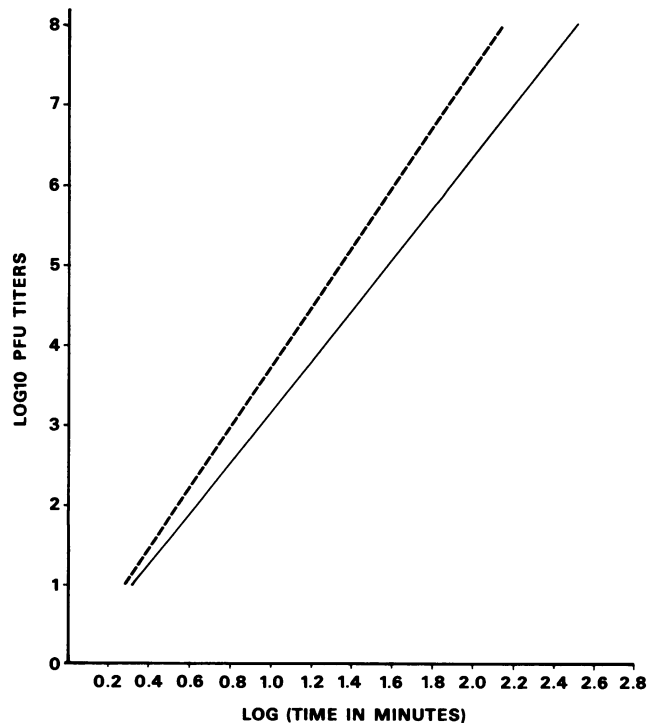


FIG. 4. Time ( $\log_{10}$  in minutes) required to inactivate a given titer of Ebola virus (----) and Lassa and Marburg viruses (—).

only at 60°C. The rate of inactivation was best described as a linear function of the log<sub>10</sub> of the time that the virus was exposed to that temperature. We plotted, as a linear regression, the log<sub>10</sub> of the titer of surviving virus against log<sub>10</sub> of the time (log<sub>10</sub> minutes at 60°C) (Fig. 1, 2, and 3) to calculate the rate of inactivation for each of the viruses. The rate of inactivation (log<sub>10</sub> virus PFU per log time) for each of the viruses was: Lassa virus, -3.19; Ebola virus, -3.73; and Marburg virus, -3.19. The times required to inactivate 5 logs PFU/ml of Lassa, Ebola, and Marburg viruses in serum were 37, 22, and 37 min, respectively (Fig. 4).

A common method for determining the leukocyte (WBC) count is to dilute the blood 1:100 in 3% acetic acid (Unopette; Becton Dickinson and Co., Paramus, N.J.), which lyses the erythrocytes and adjusts the concentration of WBCs for accurate counting. After a 15-min exposure to the acetic acid, we found that the high concentrations of Ebola, Lassa, and Marburg viruses in blood were inactivated.

We determined that holding serum at 60°C for 1 h did not alter the values of serum sodium, potassium, chloride, glucose, and blood urea nitrogen (data not shown).

Although  $1.27 \times 10^6$  rads of gamma radiation has been shown to be an effective dose for inactivating Lassa, Ebola, and Marburg viruses in serum (4), this method of inactivation is not acceptable for serum or plasma to be tested for enzyme levels, prothrombin time, or partial thromboplastin time. The amount and rate of decrease in enzyme activity caused by gamma irradiation was unpredictable and did not appear to be related to the level of enzyme present before irradiation (data not shown). Likewise, there was not a clearly defined relationship between initial clotting time and the percent increase in clotting time after gamma irradiation as measured by the prothrombin time and activated partial thromboplastin time tests (data not shown).

## DISCUSSION

Heating has long been recognized as an effective method of inactivating viruses. The kinetics of the inactivation of a virus suspension *in vitro* in the 50 to 60°C temperature range are usually determined by the inactivation of the most thermolabile proteins that are essential for infection. Temperatures higher than 60°C are required to denature nucleic acid (3). Many factors affect the thermal death time and must be considered; these include the presence of salts and organic compounds, the pH of the suspending solution, the density of the suspension, and the presence of other microorganisms in the suspension. Serum proteins have been shown to protect virus from thermal inactivation (5). We have determined survival curves and inactivation curves for Lassa, Ebola, and Marburg viruses in human serum; however, thermal inactivation is only acceptable when assaying for thermostable blood components such as electrolytes, glucose, and blood urea nitrogen.

Although Ebola and Marburg viruses are proposed to belong to the same family, Filoviridae (9), and are similar in size and protein structure, their rates of inactivation at 60°C were slightly different. Lassa virus, which is much smaller than Marburg and belongs to the Arenavirus group (2), had an inactivation rate equal to that of Marburg virus. Based on this observation, one cannot assume that related viruses will be inactivated at similar rates, and caution must be used in selecting the thermal inactivation conditions for specimens containing unknown viruses. Although we have rarely found virus concentrations exceeding  $10^5$  PFU/ml of blood from

patients viremic with Lassa, Ebola, or Marburg virus, we have established the procedure of heating at 60°C for 60 min serum that is suspected of being infected with any of these viruses, which allows an added margin of safety. Similar inactivation results for Lassa virus in human sera have been reported (10). Heating at 60°C for 1 h has also been recommended for Marburg virus (1), but the viral inactivation was not performed in the presence of serum.

Gamma irradiation has proven to be a valuable method of virus inactivation in our laboratory for many applications; however, many clinical laboratories may not have access to a <sup>60</sup>Co gamma cell. In earlier studies (Mitchell, unpublished data), we determined that the WBC count was not adversely affected by gamma irradiation; however, we sought an inactivation method that would be readily available in all hospital laboratories. A common diluent for WBC counting is 3% acetic acid (pH 2.5). The spatial arrangement of polypeptide chains within many viral proteins is known to be altered by lowering the pH of the suspending solution, thus denaturing the protein (6). We determined that a 15-min exposure to 3% acetic acid was sufficient to inactivate Lassa, Ebola, and Marburg viruses. The inactivated dilution of blood can then be placed on a hemacytometer, and the WBCs can be counted outside of a P-4 containment facility.

The wide variation in ratios of preinactivation to postinactivation values for enzyme concentrations and clotting times of plasma exposed to gamma radiation may be explained by the many factors affecting enzyme function, including pH, aggregation of protein, and denaturation through loss of tertiary structure (6). The composite of these factors, which varies from serum to serum, is undoubtedly responsible for the irregular relationship between radiation dose and enzyme degradation. It was therefore not possible to establish standard curves of enzyme degradation and radiation dose for use in estimating the original enzyme level in a serum that has been inactivated by gamma radiation.

Filtration was not considered as a practical method for inactivating viruses in serum to be used for clinical assays. For many serum analyses, the effects of filtration regardless of pore size must be defined to avoid misleading results. Removal of particles from serum by filtration through a cellulose ester filter is not purely mechanical. The electrochemical forces between the membrane filter and the particles or substances are also responsible for filter retention (13), as well as the tendency for some proteins to form aggregates (7). A membrane filter with a pore size small enough to ensure complete removal of virus particles, therefore, alters certain serum enzyme levels (Mitchell, unpublished data).

A hospitalized patient suspected of being infected with Lassa, Ebola, or Marburg virus or any class 4 viral agent presents a dual problem for the clinical laboratory. First, specimens from these patients must be handled in such a manner that the risk of exposure to laboratory personnel is eliminated. Often the most practical solution is inactivation of the virus. Second, accurate laboratory results must be promptly available to the physician to be of value in diagnosis and treatment. Unfortunately, the mechanisms involved in virus inactivation also adversely affect many blood components. We have defined three methods of inactivation for Lassa, Ebola, and Marburg viruses and have shown the effects of these methods on various blood components. In our laboratory, we have adopted the method of thermal inactivation for serum electrolytes, glucose, and blood urea nitrogen. WBC counts are determined manually after diluting the blood in 3% acetic acid. We believe these methods to

be suitable for use in any diagnostic laboratory. At present, we have not found a suitable means of viral inactivation that does not affect most enzyme levels and coagulation factors in human blood; therefore, these assays must be performed under maximum containment conditions, which can be attained in a portable containment laboratory.

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