The disease progression associated with pirital virus infection in the Syrian golden hamster

Eric M. Vela*, Katherine Knostman, Richard Warren, Jennifer Garver and Rachelle Stammen

Battelle Memorial Institute, 505 King Avenue Columbus, OH 43201-2693, United States.

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Arenaviruses are negative strand RNA viruses that can cause disease and hemorrhagic fever in humans. Typically, research with the hemorrhagic fever causing - arenaviruses requires working in a Biosafety level (BSL)-4 environment which increases the time and cost of testing vaccine and therapeutics. Therefore, we have expanded on the previously described pirital virus (PIRV) animal model for human arenaviral hemorrhagic fever viruses. The PIRV animal model can be used in a BSL-3 laboratory to define disease manifestations or “triggers” that can be used in the testing of the efficacy of potential antivirals in a therapeutic mode. Pirital virus (PIRV) is a new world arenavirus that is considered a BSL-3 non-human pathogen. Infection of the Syrian golden hamster with PIRV leads to hemorrhagic fever manifestations. In this study, we show that intraperitoneal infection of female Syrian golden hamsters (13 -15 weeks of age), implanted with telemetry units, with PIRV leads to a specific disease progression resulting in hemorrhagic fever signs, viremia, viral titers in specific tissues, and mortality. Additionally, analyses of the core body temperature telemetry data demonstrate that the core body temperature raises 24 - 36 h post infection and the normal diurnal temperature pattern is disrupted. Lastly, visible signs of neurologic disease were observed, which correlates to the presence of lesions and necrosis within the brain. In all, this study has led to a description of the disease progression associated with an arenavirus hemorrhagic fever model and describes the “trigger” that can be potentially used to test the efficacy of potential antivirals in a therapeutic setting.

Key words: Arena virus, pirital virus, hemorrhagic fever.

INTRODUCTION

Arenaviruses are rodent-borne, enveloped RNA negative-strand RNA viruses. There are at least 22 arenaviruses that have been identified (Charrel et al., 2002); however, new arenaviruses are continually being identified (Briese et al., 2009). Some of these viruses have the ability to cause a wide spectrum of human disease including hemorrhagic fever manifestations (Peters, 2002). Lassa virus (LASV) is an Old world arenavirus and is the most prevalent hemorrhagic fever arenavirus, infecting a quarter of a million individuals in endemic regions of West Africa annually (McCormick et al., 1987). The New world arenaviruses which cause hemorrhagic fever in humans include Junín virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), and Sabiá virus. All of these New World arenaviruses are category A priority pathogens and classified as HHS (Health and Human Services) Select Agents (Borio et al., 2002) and require BioSafety Level (BSL - 4) laboratories for study in the United States (CDC/NIH, 2007). Humans infected with the hemorrhagic fever - causing New world arenaviruses commonly develop fever, malaise, myalgia, and fatigue. Advanced stages of the disease may result in petechial rashes, hemorrhaging, and neurologic symptoms such as delirium, incoordination, tremors, and hyporeflexia with a high incidence of death. Working in a BSL- 4 environment presents many challenges which increase the time and cost associated with discovering therapeutic drugs. The use of a New world arenavirus requiring BSL- 3 containment would provide a more cost...
effective approach for identifying new drugs to treat hemorrhagic fevers. *Pichinde virus* (PICV) causes hemorrhagic fever manifestations in guinea pigs and has been used as a surrogate to study LASV pathology (Aronson et al., 1994; Jahrling et al., 1981; Zhang et al., 1999). However, PICV may not be suitable as a surrogate for the pathogenesis of the New world arenaviruses that cause hemorrhagic fever because hemorrhaging is not a major component of the PICV - guinea pig model, but does occur more often in human disease caused by the New world arenaviruses when compared to Lassa fever. Furthermore, infection of guinea pigs with PICV classically results in unimpressive histopathology. Pirital virus (PIRV) infection of the Syrian golden hamster is another animal model that can be used in a BSL-3 laboratory environment to study hemorrhagic fever pathogenesis since this model is primarily used as a surrogate to study LASV pathogenesis. PIRV is a New world arenavirus that is equivalent to a human pathogen that requires BSL - 3 containment and was originally isolated in the Municipality of Guanarito in Venezuela in 1994 from a cotton rat (Fulhorst et al., 1997). Infection of the Syrian golden hamster (5 - 6 weeks of age) with PIRV leads to hemorrhagic fever manifestations and mortality (Sbrana et al., 2006; Xiao et al., 2001). In this report, we describe the disease progression associated with PIRV infection in the Syrian golden hamster (13 - 15 weeks of age) and demonstrate that infection leads to necrosis, inflammation and/or lesions in the brain, kidneys spleen, liver, pancreas, lymph nodes, and intestinal lymphoid tissue. Furthermore, we monitored core body temperature with implanted telemetry units which provided body core temperature changes in real time. Infection with PIRV resulted in an increase in core body temperature and the loss of normal diurnal temperature rhythms. We demonstrate that the Syrian golden hamster hemorrhagic fever model may be used to define the disease progression associated with arenaviral hemorrhagic fevers and may be used as a surrogate to test the efficacy of various antivirals in a prophylactic and/or therapeutic model.

**MATERIALS AND METHODS**

**Animal studies**

Eight female Syrian golden hamsters (*Mesocricetus auratus*) (13 - 15 weeks of age at the beginning of study) were obtained from Charles River Kingston (Kingston, NY) and randomly assigned into two groups: Group 1 consisted of 6 animals and the Control Group consisted of 2 animals. All Animals were surgically implanted with the radiotelemetry PhysioTel TA series transmitter from Data Sciences Intl. (Arden Hills, MN) approximately 40 days prior to infection. Temperature data were recorded every 15 min in every animal. Animals were anesthetized prior to removal from the housing units for general observations on study days -2, 0, 2, 5, and 6 (day 6 for the control animals). The hamsters were individually housed in isolator cages and allowed food and water *ad libitum*. The animals were challenged with PIRV (10^5 pfu) in 1 mL of RPMI with 1% fetal bovine serum (FBS). The control animals were subject to a mock challenge consisting of a media (non - virus) control. The study protocol and all associated study procedures were approved in accordance to the guidelines by the Institutional Animal Care and Use Committee at the Battelle Memorial Institute. All work involving infected animals or virus was performed in the BSL - 3 laboratory.

**Virus**

PIRV (VAV - 488) was obtained from Dr. Robert B. Tesh at the University of Texas Medical Branch in Galveston, TX. The virus was passaged up to two times in Vero cells using RPMI 1640 media (Gibco) plus antibiotics (diluted 1:100). PIRV was diluted in RPMI with 1% (FBS) for challenge.

**Plaque assay**

Plaque assays were performed as previously described (Vela et al., 2007; Vela et al., 2008). Briefly, Vero E-6 cells were seeded at a density of 2.5 - 3.0 x 10^5 cells/mL. Samples of the virus were diluted in media and plated in duplicate at 37.0°C (5% CO2) for 20 min. A methyl-cellulose overlay containing 2x EMEM, antibiotics, and non - essential amino acids was added to the inoculum. Samples were further incubated at 37.0°C for 96 h followed by fixing and staining in a crystal violet solution containing formaldehyde for 20 min prior to visual analyses.

**Pathology studies**

A complete necropsy was performed on all hamsters. Tissues were fixed in 10% neutral buffered formalin and 5 - micron sections were prepared for routine hematoxylin and eosin staining. Microscopic examination was conducted by a board - certified veterinary pathologist.

**Tissue harvesting**

Tissues were collected in sterile 1x PBS and homogenized and centrifuged (2000 x g, 5 min at 4°C) prior to collection of the supernatant. Plaque assays were conducted to measure viral titers on the lung, heart, brain, pancreas, tissue, liver, spleen, kidney, adrenals, lymph nodes, and intestines.

**Blood sampling**

Serum samples were obtained by retro - orbital puncture and centrifuged at 1300 x g, 10 min, 4°C and assayed for viremia. Terminal samples were only obtained from animals that were euthanized due to moribundity.

**RESULTS**

**Disease progression in hamsters Infected with PIRV**

Syrian golden hamsters carrying telemetry units to
measure body temperatures were infected with PIRV \(10^5\) PFU. Intraperitoneal (i.p.) infection of the hamsters with PIRV led to a consistent disease progression (Figure 1). Elevated temperatures were observed in all animals’ 24 - 36 h post - infection with losses in body weights in all animals by 48 h post - infection. All infected animals were lethargic and exhibited huddled posture, ruffled fur, and a petechial rash by day 5 after infection. Epistaxis and ecchymoses were also observed in the animals 5 - 6 days after infection. Ocular hemorrhaging occurred in half (3/6) of the animals 6 - 7 days post - infection and the first deaths occurred 6 days after infection (Figure 2). By day 7, half of the infected animals had succumbed to infection. The remaining animals began to exhibit rectal hemorrhaging and exhibited neurological signs of disease including, tremors, loss of balance, seizures, and hind limb paralysis. All infected animals had succumbed to disease 8 days post - infection.
PIRV infection in hamsters results in weight loss and an increase in core body temperature

All animals were weighed one day prior to infection; this weight was treated as the baseline body weight. A decrease in body weights was observed 48 h post-infection in all PIRV-infected animals (Figure 3a). The four animals that died on day 6 or 7 post-infection lost between 15.0 and 17.6% of their baseline body weight. The two animals that died on 8 days post-infection lost 16.8 and 22.1% of their baseline body weight. The control animals maintained or gained weight during this time. The body temperature of the animals, collected from the surgically implanted telemetry units, was continuously monitored for 7 days prior to infection and for 8 days after infection. The body temperatures of all of the animals (PIRV-infected and control groups) were averaged and are presented in Figure 3b. A regular diurnal temperature pattern was observed in all animals prior to infection. PIRV infection led to a deregulation of the normal diurnal temperature pattern and elevated temperatures 24-36 h post-infection. Elevated core body temperatures remained elevated for 120 h (5 days) post-infection, after which the animals began to succumb to disease which was marked by a decrease in core body temperatures (Figure 3b). The temperature spikes observed on days 2, 0, 2, and 5 were associated with observations where the animals were removed from their housing, thus causing the telemetry unit to miss temperature measurements (the control group underwent an additional observation on study day 6). Additionally, the lower temperatures may also be due to use of an anesthetic, which causes a decrease in core body temperatures before stabilization. In all, infection of the Syrian golden hamsters with PIRV leads to a loss in body weight and elevated core body temperatures and a disruption of the natural diurnal temperature patterns.

PIRV infection in hamsters results in viremia and viral loads in the tissue

PIRV was recovered from several tissues including the lymph nodes, brain, liver, spleen, kidney, heart, intestines and lungs (Figure 4a). The liver and spleen carried the highest viral loads \((1.5 \times 10^5 \text{ and } 4.2 \times 10^5 \text{ pfu/mL, respectively})\). The heart, lungs, lymph nodes, and kidneys also contained higher amounts of virus (between \(1.7 \times 10^5 \text{ and } 7.3 \times 10^4 \text{ pfu/mL}\), while low viral titers were associated with the brain and intestines. Viral titers in the brain are significant findings since previous studies have not reported measureable virus in the brain. A measurable viremia was detected two days post-infection and persisted until the death of the animal (Figure 4b). The viral load increased in sera with time and the highest viremia was observed at the time of death of the animals.

Pathology

Complete necropsies were performed on all study animals. Gross lesions, consisting of dark or red discolored areas, microscopic hemorrhage, inflammation, necrosis, and mineralization were associated with multiple tissues in the PIRV-infected hamsters including the brain, small and large intestines, spleen, kidneys, liver, lymph nodes, lungs, skin, and skeletal muscle. Enlarged bronchial and mediastinal lymph nodes were also present. Lesions were similar in severity and incidence in the tissues of the PIRV-infected hamsters; however, the most affected organs appeared to be the liver, lung, brain, spleen, heart and adrenal glands. Necrotic and mineralized hepatocytes and interstitial pneumonia were observed throughout the entire liver and lung, respectively, of all of the PIRV-infected hamsters (Figures 5a-c). Coagulation necrosis was associated with lesions observed in the splenic red pulp (Figure 5d) and cardiac myofiber necrosis and mineralization were also observed in infected hamsters (Figure 5e). Microscopic lesions occurred in the brains of the PIRV-infected hamsters; however, these lesions appeared to be mild. Necrotic cells were also associated with different sections of the brain and hemorrhaging was found in the meninges and perivascular regions (Figure 5f-i). Hemorrhage and accumulation of fibrin in the cerebral cortex of the brain were detected in the Virchow-Robin space of PIRV-infected hamsters (Figure 5j), while hemorrhage and degeneration and necrosis of the Purkinje cells in the cerebellum were also observed (Figure 5k). Perivascular edema of the cerebral cortex was also associated with PIRV infection (Figure 5l). Mock-control animals did not demonstrate any of the necrosis, inflammation, or hemorrhaging observed in the infected animals (Figures 5m and 5n). In all, infection of the hamsters with PIRV leads to gross lesions, microscopic hemorrhage, inflammation, necrosis, and mineralization in multiple tissues.

DISCUSSION

Diagnosis of arenaviral disease in humans is often associated with late disease manifestations such as neurologic and hemorrhagic symptoms (de Manzione et al., 1998; McCormick et al., 2002). Thus, very little information exists describing the early stages of arenaviral disease pathogenesis in humans. However, infected individuals commonly develop fever, malaise,
Figure 3A. PIRV infection in hamsters results in weight loss. (A) Animals were weighed on Study Day -1 (baseline), 2, 5, 6, 7, and 8. All animals were infected with PIRV on Study Day 0. The red crosses represent the day each animal died.

Figure 3(B). Animals were implanted with telemetry units and the temperatures were recorded every 15 min from Study Day -7 until the end of the study. The temperature averages of the animals in each group were averaged. Temperature spikes observed on days -2, 0, 2, and 5 were associated with daily observations (the control group underwent an additional observation on Study Day 6).
Figure 4a. PIRV infection in hamsters leads to measurable viral loads in specific tissues and viremia. (a) Samples of lymph nodes, brain, liver, spleen, kidney, heart, intestines, and lungs were harvested from all dead animals and viral loads were calculated using plaque assay analyses.

Figure 4b. Blood sera were collected from each animal and viremia was determined by plaque assay analyses. The viral titers of each tissue were averaged and viremia values from all animals were averaged for Study Day 2 and 5. The terminal samples refer to the day of the death of each animal.
Figure 5. PIRV infection leads to gross lesions, microscopic hemorrhage, inflammation, necrosis and mineralization in the liver, lung, spleen, heart, and brain. (A) Liver - hepatocellular vacuolization, necrosis and accumulation of neutrophils and fibrin in the sinusoids of a PIRV-infected hamster (H and E-40x magnification). (B) Liver-hepatocellular vacuolization, necrosis and mineralization of necrotic hepatocytes in a PIRV-infected hamster (H and E-40x magnification). (C) Lung - interstitial pneumonia in a PIRV-infected hamster. Interstitial blood vessels and alveolar septae are mildly expanded by fibrin, necrotic cellular debris and neutrophils (H and E-40x magnification). (D) Spleen - acute coagulation necrosis of red pulp with abundant fibrin and scant neutrophilic accumulation in a PIRV-infected hamster. The periaorteriolar lymphoid sheath on the right is also depleted (H and E-20x magnification). (E) Heart-coagulation of individual myocardial fibers with mineralization in a PIRV-infected hamster (H and E-40x magnification). (F) Brain, cerebral cortex-endothelial and glial cell necrosis in the brain of a PIRV-infected hamster (H and E-40x magnifications). (G) Brain, hippocampus-neuronal necrosis in the hippocampus of a PIRV-infected hamster (H and E-40x magnifications). (H) Brain, thalamus-hemorrhage and perivascular accumulation of mononuclear cells in the thalamus of a PIRV-infected hamster (H and E-20x magnification). (I) Brain, thalamus - hemorrhage and focal neuropil necrosis in the thalamus of a PIRV-infected hamster (H and E-40x magnifications). (J) Brain, cerebral cortex-hemorrhage and accumulation of fibrin and a few neutrophils in the Virchow-Robin space of a PIRV-infected hamster (H and E-20x magnifications). (K) Brain, cerebellum-hemorrhage and degeneration and necrosis of Purkinje cells (arrows) in the cerebellum of a PIRV-infected hamster (H and E-40x magnification). (L) Brain, cerebral cortex-perivascular edema at the gray-white junction of the cerebral cortex in a PIRV-infected hamster. Many pyknotic nuclei are present in the ependymal neuropil, likely representing both glia and neurons. One necrotic endothelial cell is noted (H and E-40x magnification). (M) Control cerebellum from a mock-infected control animal (H and E-20x magnification). (N) Control cortex from a mock-infected control animal (H and E-20x magnification).
myalgia, and fatigue 7 - 18 days after infection. Petechial rashes, hemorrhaging, and neurologic symptoms such as delirium, incoordination, tremors, and hyporeflexia are commonly associated with the advanced stages of disease, in addition to respiratory distress, shock, vascular permeability, signs of encephalopathy, seizures, and coma. Interestingly, 15% of people who recover from Lassa hemorrhagic fever experience deafness. Arenaviral loads in patients who succumb to disease are generally higher than survivors. Immunosuppression may contribute to higher viral loads in fatal cases.

The previously described PIRV Syrian golden hamster model (Sbrana et al., 2006) can be used to define the early phase of the arenavirus disease progression. We have expanded this model to define both the disease progression and suggest that an elevated temperature and viremia in PIRV - infected animals may be used as “triggers” to test potential therapeutic treatments. The trigger would be used as the metric that correlates to the start of treatment. Our data demonstrates that infection of female Syrian golden hamsters with PIRV leads to a disease progression consisting of elevated temperatures, loss of body weight, lethargy, formation of petechial rashes, huddled posture and ruffled fur, epistaxis, and ocular hemorrhaging. Animals that survived to the later stages of disease exhibited neurological signs of disease such as whole body tremors, loss of balance, shaking, and hind limb paralysis, in addition to rectal hemorrhaging. Overall, disease progress is similar to what has been previously published (Sbrana et al., 2006). Previously clinical observations included lethargy and anorexia. Some of the hamsters demonstrated petechiae, epistaxis, and hind limb paralysis. All hamsters, in this previous study all died nine days after challenge. These results are similar to what was observed in this current study. Additionally, previous studies and the current study both demonstrated increased ALT and AST levels, as well as similar coagulation changes. This current study demonstrated that PIRV infection also led to a loss of body weight. Infected animals lost an average of 17.3% of their baseline body weight, while the control uninfected animals gained weight throughout the study. Disruption of the natural diurnal temperature rhythm resulted from PIRV infection. Temperatures (normal temperature is approximately 37.5°C) elevated 24 - 36 h post infection and culminated with a marked period of lower temperatures prior to the death of all animals. The temperature spikes observed may be due to the use of the anesthetic and removal of the animals from their telemetry – measured environments. Anesthetics will cause a decrease in temperature in hamsters, which may take 4 - 6 h to raise and normalize. This is evident in the temperature data from the PIRV - infected animals (Days 1-3). Temperatures were increased on Day 1 post - infection and began to decrease prior to the temperature spike. Similarly, the temperatures began to rise after the temperature spike on day 2 and elevated to temperatures above what was considered normal. The lowering of the temperatures can be observed in both the infected and control animals that were anesthetized. Elevated temperatures appeared to correlate with viremia 24 - 48 h post - infection. Thus, these two parameters can be used, in concert, as triggers for a therapeutic treatment model. PIRV infection in these hamsters resulted in 100% mortality by day 8 post - infection.

High viral titers were observed in the liver and spleen, while lower titers were detected in the lymph nodes, heart, kidney, and lungs. The presence of virus in the brain, albeit at low but detectable levels, has not been previously demonstrated. Viral titers in the brain may be due to the age of the animals, since previous studies used hamsters 5 - 6 weeks of age, which is the only major difference associated with the studies. The animals that contained viral loads in the brain correlated with microscopic hemorrhage and lesions and animals that presented with neurologic signs of disease such as tremors, loss of balance, and hind limb paralysis. Necropsies of the PIRV - infected animals revealed gross lesions, microscopic hemorrhage, inflammation, necrosis, and mineralization in the brain, small and large intestines, spleen, kidneys, liver, lymph nodes, and lungs, skin, and skeletal muscle, which is in agreement with the viral titers observed in the specific tissues. Intestinal hemorrhaging was also observed at the time of necropsy and the spleens in the PIRV - infected animals were noted as friable. Based on the necrosis and mineralization results, hepatocytes and endothelial cells may likely to be viral targets of infection. In all, this data is similar to what has been previously observed (Sbrana et al., 2006; Xiao et al., 2001); however, these studies did not report any mineralization in the tissues and no lesions in the heart, brain, kidney, or intestines. Several arenavirus infection animal models have been described; however, most require a BSL - 4 laboratories and techniques. GTOV and LASV infection has previously been described in guinea pigs (Hall et al., 1996; Jahrling et al., 1982). GTOV infection in guinea pigs results in morbidity, necrosis of the gastrointestinal epithelium, interstitial pneumonia, and platelet thrombi in some blood vessels. Viral antigen was demonstrated in lymphoid tissues, macrophages, and endothelial cells. Infection of guinea pigs with GTOV results in a lethal disease marked by pulmonary and adrenal hemorrhage and bone marrow depletion.

Similarly, LASV infection in inbred guinea pigs results in viremia, viral titers in tissues, histological lesions, and morbidity and mortality. Both models are valid animal models to study human disease. Additionally, LASV infection in rhesus macaques results in clinical signs of
disease that are typical and similar to those observed in humans (Jahrling et al., 1980; Lange et al., 1985).
Infection results detectable viremia 5 - days post-infection, weight loss, and hypothermia and hypotension prior to death. Viral titers and lesions are observed in the liver, spleen, adrenals, kidneys, pancreas, heart, lung, and brain. Thrombocytopenia and elevated AST and ALT levels have also been observed. As for coagulation, APTT is consistently abnormal during the terminal phase of disease, which may be marked by vascular collapse. Studies with primates are expensive and these studies require a BSL - 4 environment, thus a small animal non – BSL - 4 surrogate model would be useful in antiviral and vaccine therapeutics. The PIRV - hamster model provides such a model and the pathogenesis is similar to those described. We demonstrate that real - time telemetry of core body temperature can be used as a clinical indication of infection with PIRV and is a potential therapeutic trigger. The results demonstrate that elevated temperatures and the presence of viremia can be used as parameters for the start of a drug treatment regimen in a therapeutic hemorrhagic fever model since these parameters are measurable early in the disease process.
Because of similarities with disease signs associated with infection of both the Old world and New world arenaviral hemorrhagic fever viruses, this surrogate system can be used to evaluate the efficacy of potential antivirals, vaccines, and therapeutics. In conclusion, this study has led to a description of a hemorrhagic fever model to study arenavirus hemorrhagic fever pathogenesis and can be used as a cost effective, time - efficient BSL - 3 surrogate to screen various antiviral therapeutics and/or vaccine strategies.

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REFERENCES