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REVIEW ARTICLE

Eight Years of Research Advances in Bourbon Virus, a Tick-borne Thogotovirus of the Orthomyxovirus Family

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Abstract

Bourbon virus (BRBV) was first isolated from a blood sample collected from a male patient living in Bourbon County, Kansas, during the spring of 2014. The patient later died because of complications associated with multiorgan failure. Several deaths due to BRBV infection have since been reported in the United States, and misdiagnosed cases are often undercounted. BRBV is a member of the genus *Thogotovirus* of the *Orthomyxoviridae* family, and is transmitted through the Lone Star tick, *Amblyomma americanum*, in North America. Currently, no specific antiviral agents or vaccines are available to treat or prevent BRBV infection. Several small-molecular compounds have been identified to effectively inhibit BRBV infection of *in vitro* cell cultures at the single- or sub-micromolar level. Favipiravir, an RNA-dependent RNA polymerase inhibitor, has been found to prevent death in type I interferon receptor knockout mice with BRBV infection.

Keywords: Bourbon virus, Lone Star tick, infection, antiviral agents

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INTRODUCTION

Bourbon virus (BRBV) is a member of the genus *Thogotovirus* of the *Orthomyxoviridae* family of segmented negative-strand RNA viruses [1]. Seven genera exist in the *Orthomyxoviridae* family: four types of influenza viruses (*influenza virus A, B, C* and *D*), *Quaranjavirus, Isavirus* and *Thogotovirus* (Fig 1). Many viruses in the *Orthomyxoviridae* family are important pathogens in humans and animals. The epidemic/pandemic influenza A viruses have caused hundreds of thousands of human deaths and continue to circulate and greatly threaten human lives.

In contrast to influenza viruses, thogotoviruses are transmitted mainly through tick vectors and thus are also called tick-borne viruses [3,4]. Two thogotoviruses, Thogotovirus and Dhori virus, have been reported to infect humans and cause death [5,6]. Thogotovirus circulates primarily in domestic animals, such as sheep, cattle and camels, in which it causes neurological diseases and abortion [7]. Human antibodies against Thogotovirus and Dhori virus have been identified in Europe, Asia and Africa [7,8]. Importantly, Dhori virus has been reported to cause human infections in a vector-free manner, possibly through an aerosol route [5], thus highlighting the potential to infect humans in large populations.

On the basis of available genomic sequences, thogotoviruses have been subdivided into two clusters [9-12]: Thogoto-like and Dhori-like thogotoviruses. BRBV shows a close phylogenetic association with Dhori-like thogotoviruses, e.g., Batken virus, whose emergence has been reported
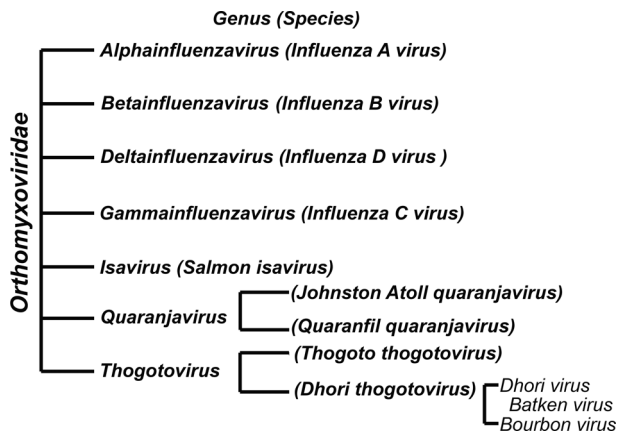
FIGURE 1 | Classification of *Orthomyxoviridae*. Seven genera of the *Orthomyxoviridae* family are listed with species shown in parentheses, according to the revised classification of *Orthomyxoviridae* (ICTV 2017) [2].

in regions throughout Africa, Asia and Europe [7,8,13,14]. Dhori and Batken viruses have been isolated from *Hyalomma* ticks, and antibodies against Dhori virus have been identified in camels, goats, horses, cattle and humans [7,8,13,14]. Interestingly, Batken virus has also been isolated from several mosquito species [14]. BRBV replicates at high levels in a variety of invertebrate and vertebrate cell lines, and produces relatively high titers after infection of all mammalian cells, a finding consistent with the known susceptibility of human hosts to BRBV infection and disease [11].

Bourbon virus infection causes human diseases

The first case of BRBV infection was reported in Kansas, USA. The virus was isolated from a blood sample collected from a male patient living in Bourbon County, Kansas, who was hospitalized at The University of Kansas Hospital in Kansas City, in the spring of 2014 [15]. The patient was a healthy man >50 years of age who had been bitten by several ticks before hospitalization. During the beginning of his illness, symptoms included nausea, weakness and diarrhea. After 2 days, he experienced fever, anorexia, chills, headache, myalgia and arthralgia. He was hospitalized after 4 days. Laboratory tests revealed leukopenia, lymphopenia, thrombocytopenia, hyponatremia, and elevated aspartate aminotransferase and alanine aminotransferase. He died 11 days after the onset of symptoms [15]. Because the patient had a papular rash on his trunk, serological and molecular testing for several known tick-borne pathogens were performed, but all were negative [15]. However, when a serum sample was inoculated in Vero cells, at 3 days post-infection, clear plaques were observed in a plaque assay. The supernatants of the inoculated culture were observed to contain filamentous and spherical virus-like particles under transmission electron microscopy; moreover, a morphology typical of virions in the family *Orthomyxoviridae*, and novel viral sequences sharing high similarity with Dhori and Batken viruses were identified [4,16]. This novel *Thogotovirus* was named BRBV after the county where the patient lived. Because of the high

viremia and identification of the virus in the serum of the patient, BRBV was believed to have been the cause of the patient's illness and death.

To date, a limited number of cases of BRBV disease have been identified in the Midwest and southern United States. Some infected people have later died. In 2015, a patient who was a resident of Payne County, Oklahoma, tested positive for neutralization antibodies to BRBV before fully recovering [17]. In June 2017, a 58-year-old woman from Missouri died from an infection of BRBV after 23 days in the hospital. The patient's symptoms and laboratory test results were similar to those described in the first case in Kansas approximately 1 week after tick bites [18]. The virus was isolated from Vero cells inoculated with a serum sample collected from the patient, and viral RNA was sequenced through next-generation sequencing, with an average of 10^4-fold coverage, and identified as BRBV [18].

BRBV is the first species of the genus *Thogotovirus* to be identified as a human pathogen in North America.

The Lone Star tick is the vector for BRBV transmission

The United States Centers for Disease Control and Prevention (CDC) performed several epidemiologic studies on BRBV infection in *Amblyomma americanum* ticks (Fig 2) and animals by using real-time PCR with BRBV-specific primer/probe sets targeting the nucleoprotein (NP) and polymerase (PB1) genes. In 2013, BRBV was found in three pools of *Amblyomma americanum* ticks from retrospective tests in 39,096 ticks from northwestern Missouri, located 240 km from Bourbon county, Kansas [17]. The BRBV infection rate (IR) per 1,000 adult ticks for all sites was 0.32. The BRBV strain isolated from tick pools shared >99.0% sequence at the amino acid level and 95.0% identity at the RNA-sequence level, with the first-identified human BRBV strain [17]. In the summer of 2015, CDC continued tick surveillance in Bourbon county and adjacent southern Linn county, Kansas. A total of 20,639 host-seeking
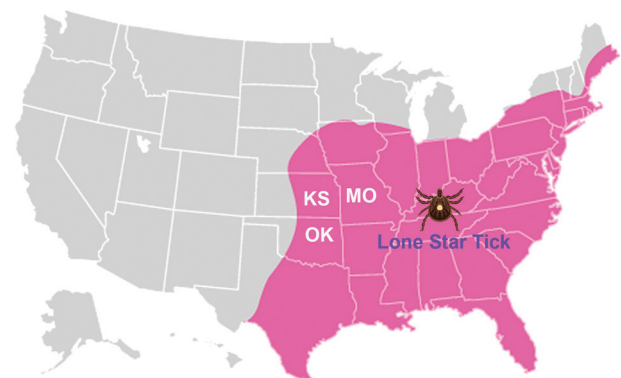

FIGURE 2 | Geographical distribution of *Amblyomma americanum*, the Lone Star tick, in the United States. The area highlighted in pink indicates the distribution of Lone Star ticks in North America, up to the upper midwestern and northeastern regions of the United States and eastern Canada. In the United States: KS, Kansas; MO, Missouri; OK, Oklahoma.

ticks representing four species were collected from 12 sites. BRBV was detected at an IR of 0.25 in the abundant and aggressive human-biting tick *Amblyomma americanum* in Bourbon county. This survey supported that *Amblyomma americanum* is a vector of BRBV [19]. In June 2016, the same group collected a total of 14,193 ticks representing four species from four sites in eastern Kansas, including the sites where BRBV was detected in 2015. Unexpectedly, all pools tested negative for BRBV [20]. Thus, *Amblyomma americanum* might infrequently encounter viremic vertebrate hosts of BRBV, or BRBV might be inefficiently passed vertically from infected female ticks to their offspring.

Moreover, in a surveillance study of animals for evidence of BRBV infection, plaque-reduction neutralization testing (PRNT) was used to detect anti-BRBV antibodies in animal sera and plasma specimens of white-tailed deer (*Odocoileus virginianus*), raccoon (*Procyon lotor*), Virginia opossum (*Didelphis virginiana*), and various other mammals and birds from northwest Missouri. A high seroprevalence in wild animals, raccoons (50%) and white-tailed deer (86%), and a low rate in domestic dogs (seroprevalence 15%) and horses (seroprevalence 4%) were detected [21]. In North Carolina, where the Lone Star tick is abundant, 18 of 32 (56%) white-tailed deer have been found to be BRBV-specific PRNT positive [22]. This study has indicated that BRBV is widespread among animals, as previously recognized, and has implicated raccoons and white-tailed deer as potential zoonotic reservoirs of BRBV.

BRBV replicates in cell lines derived from hard ticks *Amblyomma*, *Hyalomma* and *Rhipicephalus* [11]. Experimental infection of *Amblyomma americanum* with BRBV has indicated transstadial transmission of BRBV from the larval to nymphal stages, and from the nymphal to adult stages of the tick [23]. Importantly, a high rate of transmission from infected to uninfected ticks through cofeeding has been observed. Therefore, cofeeding may be an important mechanism for viral maintenance in the environment, in line with the finding of nonviremic or cofeeding transmission of Thogotovirus by *Rhipicephalus Appendiculatus* [24]. Although vertical transmission to progeny occurred at a low rate, rabbits bitten by BRBV infected ticks in active life stages or needle-inoculated with BRBV have been found to develop high titers (1:320 to 1:2,560) of 90% PRNT-positive to BRBV. Interestingly, BRBV injected rabbits have tested negative for virus through both RT-PCR and plaque assays, although serologic conversion has been observed at 42 days post-inoculation [23].

Together with the geographic location of the BRBV infection and the geographic distribution of *Amblyomma americanum* ticks [17,19], these studies strongly suggest that Lone Star ticks (*Amblyomma americanum*) widely carry BRBV and are a competent vector for BRBV transmission. The Lone Star tick is aggressive, feeds on humans, and is abundant in the states of Kansas, Missouri and Oklahoma (Fig 2). Importantly, the distribution of Lone Star ticks in North America has geographically expanded to the upper midwestern and northeastern regions of the United States and eastern Canada, owing to climatic change and land-use patterns [25]. Moreover, Lone Star ticks have become the most commonly encountered tick in Delaware [26]. Importantly, BRBV was detected in pools of tick *Haemaphysalis Longicornis* (Ixodidae), the Asian long horned tick, in several counties in Virginia [27]. Future epidemic surveillance of BRBV in ticks and animals will be necessary to assess the geographic distribution of BRBV and the natural host of BRBV.

BRBV genome and replicon construction

BRBV contains six segments of a negative-sense (-)RNA genome—*PB2*, *PB1*, *PA*, *NP*, *GP* and *M*—with sizes of 2,381, 2,200, 1,961, 1,480, 1,588 and 9,57 nucleotides, respectively (Fig 3A). These segments include genes encoding the RNA-dependent RNA polymerase (RdRP), consisting of the polymerase basic protein 2 (PB2), PB1 and the polymerase acidic protein (PA), an NP that encapsidates the -RNA genome, a glycoprotein (GP) that is involved in viral attachment and fusion, and a matrix protein (M) linking the viral envelope with the virus core [28,29].

Similarly to that of influenza virus, BRBV RNA replication follows the model of viruses in the family *Orthomyxoviridae*. The viral polymerase (P) subunits—PB1, PB2 and PA—compose the viral RNA replicase complex [30]. The viral RNA replication elements in *cis* are located at the 3´ and 5´ noncoding regions (NCR) of each viral genome [30]. With expression of the BRBV replicase complex (PB1, PB2 and PA), the nucleocapsid (NP) and a reporter (GFP or *Gaussia* luciferase, gLuc)-coding gene flanked by BRBV 3´ and 5´ NCR replicate and express GFP or gLuc. This replicon reporter has been independently established in three laboratories, and used for testing or screening of antiviral agents in a biosafety level 2 setting [18,31,32].

One of the six viral replication-essential genes (*PB2*, *PB1*, *PA*, *NP*, *GP* and *M*) is amplified from viral RNA extracted from infected cells and cloned into the plasmid pHW2000 to generate pBRBV-PA/PB1/PB2/NP/GP/M (Fig 3B). pHW2000 has been widely used in the development of a reverse genetic system of influenza viruses [33]. Next, a reporter (GFP or gLuc) cDNA is cloned into pHW2000 without the cytomegalovirus immediate-early enhancer and promoter (CMVp); this vector is denoted pBRBV-ΔCMV-GP-gLuc (Fig 3C). Then four CMVp driven PA-, PB1-, PB2- and NP-expressing plasmids (i.e., pcDNA-PA/PB1/PB2/NP), and pBRBV-gLuc are co-transfected into HEK293T cells. At 2 days post-infection, transfected cells are assayed for gLuc expression. Expression of PA, PB1, PB2 and NP replicates the BRBV-*gLuc* RNA genome, thus forming a capped viral positive sense (+)RNA genome, which can undergo translation. Because the *gLuc* gene in pBRBV-gLuc is not driven by a polymerase II promoter (e.g., a CMVp), only replication of the BRBV genome generates viral +RNA for gLuc expression (Fig 3C).

If all six pHW2000 based plasmids—pBRBV-PB2, PB1, PA, NP, GP and M (Fig 3B)—are co-transfected into cells,
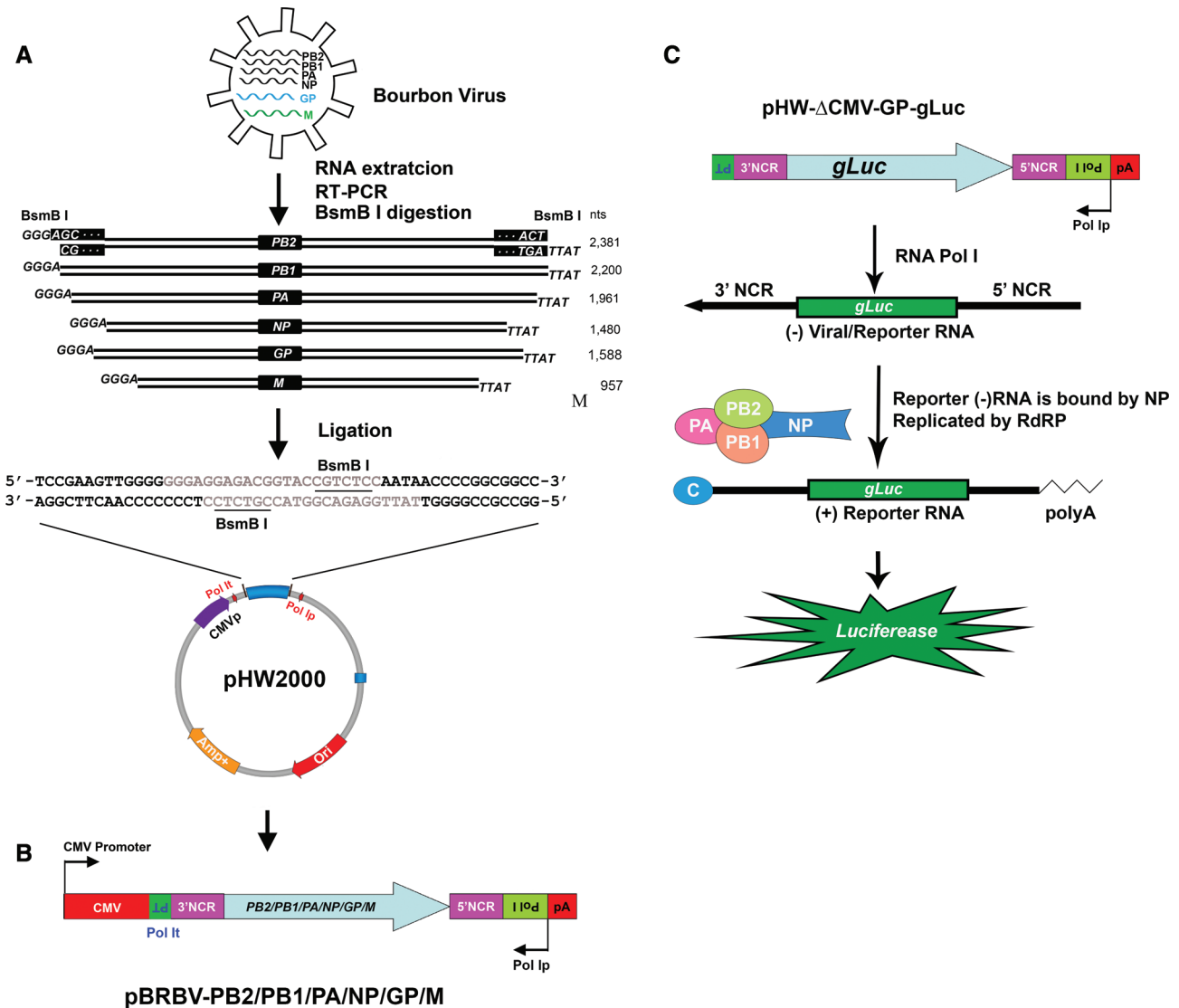
FIGURE 3 | BRBV replicon construction. (A) Schematic representation of the method for the construction of the plasmids to replicate the BRBV genome. Viral RNA was extracted from virus particles. Reverse transcription (RT)-PCR was performed with primers containing segment-specific nucleotides and sequences for the restriction enzyme BsmB I. The six viral RT-PCR fragments were digested with BsmB I and inserted into pHW2000 (linearized with BsmB I). This insertion resulted in six expression constructs, in which the viral cDNAs were precisely fused to the polymerase I promoter (Pol Ip) and the terminator (Pol It). The viral terminal sequences (AGC… and …ACT) are shown in only the PB2 segment in the black rectangles. (B) pBRBV-viral cDNA. The diagram shows the expression plasmid with a Pol Ip and a CMV pol II promoter containing viral cDNA of the PB2, PB1, PA, NP, GP or M segment. The open reading frames for the six viral proteins are flanked by the segment-specific 3′ and 5′ noncoding regions (3′ NCR and 5′ NCR, respectively; pink boxes). (C) BRBV RNA replicon reporter system. pBRBV-gLuc reporter. *Gaussia* luciferase (gLuc) gene flanked by the 3′ and 5′ NCR of BRBV is cloned into pHW2000 lacking the CMV pol II promoter. Thus, only replication of the gLuc gene by BRBV RNA-dependent RNA polymerase (RdRP) and NP protein produces gLuc +RNA for gLuc protein expression.

recombinant BRBV can be generated. Similar reverse genetic systems for influenza virus and Thogotovirus have been established and widely used to study viral replication and pathogenesis [33–38].

In vitro culture of BRBV

Vero cells (ATCC [#]CCL-81), kidney epithelial cells of the African green monkey, are mostly permissive to BRBV infection. A production level of 10^8 plaque forming units (pfu)/ml can be readily obtained (Fig 4) when Vero cells are infected with the BRBV strain (#NR–50132, the

Biodefense and Emerging Infections Research Resources Repository (BEI), NIAID, NIH), which was originally iso‐lated in Bourbon county. Diverse human cells, including the human embryonic kidney cell line HEK293T (ATCC #CRL-11268), human hepatocyte cell line Huh7, human cervical epithelial cell line HeLa (ATCC #CCL-2) and human lung epithelial cell line A549 (ATCC #CCL-185), are permissive to productive BRBV infection [11,31]. HEK293T and Huh7 cells exhibit growth kinetics sim‐ilar to those in Vero cells, but both A549 and HeLa cells show an ~2 log decrease in viral production [11,31,39].
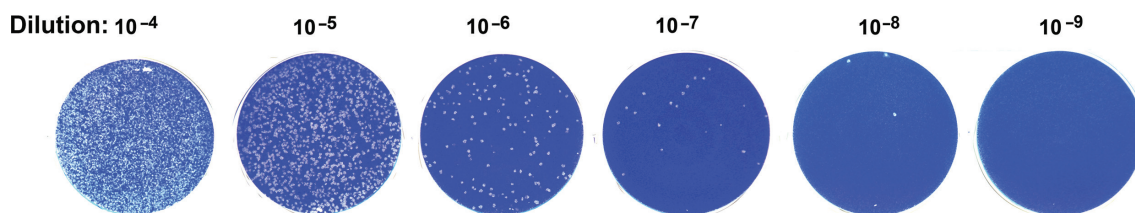
FIGURE 4 | BRBV plaque assay. A BRBV stock was diluted from 10^{-4} to 10^{-9} in Dulbecco's modified Eagle's medium (DMEM). Subsequently, 1 ml of each diluted virus was added per well of a six-well plate of Vero cells. After incubation for 1 hour at 37°C, diluted virus-containing medium was removed from each well, and 2 ml of DMEM with 10% fetal calf serum and 1% methylcellulose overlay was added to each well. At 3 days post-infection, the medium was aspirated from each well, and the cells were stained with crystal violet [31]. Plaques, foci of infected cells, are shown as white dots in the stained cell sheet (blue background).

The virus also can infect polarized human airway epithelia at a level of ~1×10^6 to 10^7 genome copies per milliliter of apical wash at 1 week post-infection. Of note, human foreskin fibroblast cells (HFF; ATCC #SCRC-1041) are not infected by BRBV, although humans are believed to be infected by tick bites on the skin. These cells may have a strong interferon (IFN) response after viral infection [40] and therefore may be resistant to BRBV infection. BRBV infection has been demonstrated to be sensitive to treatment with IFN-α2a and IFN-γ, and BRBV infection-induced IFN elicits an antiviral immunity that suppresses viral propagation [39]. BRBV replicates in cell lines derived from the hard ticks *Amblyomma*, *Hyalomma* and *Rhipicephalus*, but at a level >4 log less than that in Vero or Huh7 cells. Mosquito cell lines are poorly infected, with a yield less than 10^4 pfu/ml [11].

Animal models

Mice (CD-1) are susceptible to BRBV infection, as evidenced by seroconversion, but the infection does not cause any disease symptoms or death [11]. In C57BL/6 mice intraperitoneally infected at a high dose of 10^5 pfu per animal, BRBV does not lead to severe disease, and causes no decrease, or only a transient decrease, in body weight [12].

Type I IFN deficient mice (lacking IFN-α/β receptor expression) (IFNαAR$^{-/-}$), type I and III deficient (IFNαAR$^{-/-}$ IL28R$^{-/-}$ double knockout) mice, and type I and II IFN deficient (STAT1 knockout) (STAT1$^{-/-}$) mice support growth of BRBV-KS (the original isolate in Kansas) in liver, lung, spleen and kidney cells [39], among which kidney cells show the highest viral titers. In a study of clinical and pathologic changes, STAT1$^{-/-}$ mice with intraperitoneal inoculation of 100 pfu have been found to show the most severe clinical score and to die, whereas IFNαAR$^{-/-}$ mice have been found to be less severely affected and to recover [39]. The clinical and pathologic changes observed in STAT1$^{-/-}$ animals are compatible with the clinical manifestations, such as liver damage and acute respiratory complications, described in BRBV-infected patients [15,18]. Thus, STAT1$^{-/-}$ mice may be an ideal model to understand the replication and pathogenesis of BRBV, and to evaluate antiviral strategies. However, in another study, with intraperitoneal inoculation of a higher titer (4×10^2 pfu) of the BRBV STL strain, isolated in St. Louis, IFNAR$^{-/-}$ mice

have shown not only substantial weight loss but also 100% mortality after infection [18].

Prevention and treatment

No specific antiviral agents or vaccines have been developed to treat diseases caused by BRBV infection. IFN treatment significantly decreases viral yields in BRBV infected cell cultures. A combination of IFN-α and IFN-γ decreases the viral titer by >10^3-fold [39]. Treatment with a guanosine analog (ribavirin) and a guanine analog (favipiravir [T705]) has been found to decrease viral titer by > 1×10^6-fold in cell culture. Treatment of IFNαAR$^{-/-}$ or STAT1$^{-/-}$ mice with ribavirin (40 mg/kg) has been observed to decrease BRBV replication in organs and lead to weight loss, thus significantly delaying death in STAT1$^{-/-}$ mice. However, in another study, favipiravir has been found to protect IFNαAR$^{-/-}$ mice from lethal BRBV infection. In IFNαAR$^{-/-}$ mice, administration of 150 mg/kg of favipiravir twice daily at 3 days post-infection has been found to protected all animals against death due to BRBV infection [18]. However, in the sera of these treated animals, favipiravir has been observed to reach a concentration of 1.28 mM, thus prompting questions regarding the practical use of favipiravir to treat BRBV-infected patients. The half-maximal inhibitory concentration (IC$_{50}$) of favipiravir against BRBV infection in Vero cells is 310 μM [17], and that in HEK293T cells is 64.48 μM [32].

In an effort to identify antiviral agents against BRBV infection, myricetin, a flavonoid, has been identified to effectively inhibit BRBV infection, with an IC$_{50}$ of 4.6 μM in HEK293T and 20 μM in Vero cells [31], thus suggesting that myricetin may be a more potent inhibitor of BRBV infection than favipiravir. Myricetin is a common plant-derived flavonoid, which exhibits a wide range of activities, such as antioxidant, anticancer, antidiabetic, anti-inflammatory and antimicrobial activities [41]. Notably, myricetin is an active inhibitor of the helicase activity of nsp13 of the severe acute respiratory syndrome coronavirus (IC$_{50}$=2.71 μM) [42,43], and is a strong inhibitor of the reverse transcriptase of retroviruses [44]. More importantly, myricetin does not exert clear cytotoxicity in Vero or HEK239T cells [31], as well as normal breast epithelial cells [42,43]. The half-maximal cytotoxic concentrations (CC$_{50}$) of myricetin in HEK293T and Vero cells are 537 μM and >1 μM, respectively [31].

Screening of an antiviral compound library of 596 bioactive antiviral compounds with the BRBV replicon reporter has identified two dihydroorotate dehydrogenase (DHODH) inhibitors, hDHODH–IN–4 and brequinar, with high potency in inhibiting BRBV infection in HEK293T cells, with an IC_{50} of 0.33 and 0.07 µM, respectively [32]. hDHODH–IN–4 and brequinar also show pan-inhibition of replicon replication of other orthomyxoviruses, including human influenza A, swine influenza D virus and Thogotovirus, in a nanomolar range. Unfortunately, the cytotoxicity of these compounds has not been examined.

Overall, potent antiviral compounds against BRBV infection have great promise in treating BRBV infection in patients. Examining the antiviral activity of myricetin, hDHODH–IN–4 and brequinar in IFNαAR$^{-/-}$ or STAT1$^{-/-}$ mice exposed to different BRBV isolates should prove interesting.

Future directions

Since the identification of BRBV in 2014, molecular epidemiology studies have confirmed that the Lone Star tick is the vector transmitting BRBV. Lone Star ticks (*Amblyomma americanum*) are present in North America up to the upper midwestern and northeastern regions. In the future, surveys of BRBV or related viruses in hard ticks in the genus *Amblyomma* should be performed in other regions, in Eurasia, Africa, Australia and Asia (https://web.archive.org/web/20100922170634/http://www.kolonin.org/4.html). A new Dhori–like thogotovirus, Oz virus, has recently been isolated from the hard tick (*Amblyomma Testudinarium*) in Ehime, Japan [10]. Phylogenetic analyses have indicated that Oz virus is most closely associated with BRBV in GP- and M-encoding genes. Interestingly, like BRBV, Oz virus in C57BL/6 mice does not cause severe disease, in contrast to other thogotoviruses [12]. Human infection with Oz virus has not been reported.

With the establishment of the BRBV replicon, high–throughput screening of small-molecular compounds preventing replication of the replicon, and further validation of the candidates in cell and animal models of viral infection will be the next steps toward developing antiviral agents against BRBV infection. The structure of effective compounds against RdRP will be necessary for further structure–based design of antiviral agents.

In addition, understanding of the tropism of the viral infection through identification of the host receptor or factors that mediate BRBV entry will be crucial. Much remains to be studied regarding the life cycle of BRBV.

CONFLICTS OF INTEREST

The authors declare that no conflicts of interest exist.

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