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Molecular, serological and *in vitro* culture-based characterization of Bourbon virus, a newly described human pathogen of the genus *Thogotovirus*

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Abstract

Background—In June of 2014, a previously healthy man from Kansas with a recent history of tick exposure died from complications related to an illness marked by fever, thrombocytopenia and leukopenia. An isolate was derived from the blood of this patient during the course of diagnostic testing. This isolate was subsequently identified as a novel orthomyxovirus of the genus *Thogotovirus* by next generation sequencing and was named Bourbon virus after the patient's county of residence.

Objectives—To support research and diagnostic aims, we provide a basic description of Bourbon virus at both the molecular and serological levels. Furthermore, to preliminarily identify potential host and vector range associations we have characterized the growth kinetics of Bourbon virus in a variety of vertebrate and invertebrate cell lines.

Study design—Bourbon virus was subjected to next generation-high throughput sequencing, phylogenetic, and basic structural protein analyses as well as 2-way plaque reduction neutralization assays. Also, we inoculated a variety of cell types with Bourbon virus and evaluated the growth kinetics by determining viral titers in the supernatants taken from infected cells over time.

Results—Bourbon virus possesses 24–82% identity at the amino acid sequence level and low serological cross-reactivity with other *Thogotoviruses. In vitro* growth kinetics reveal robust replication of Bourbon virus in mammalian and tick cells.

Conclusions—Molecular and serological characterizations identify Bourbon virus as a novel member of the genus s*Thogotovirus*. Results from cell culture analyses suggest an association between Bourbon virus and mammalian and tick hosts.

Keywords

Bourbon virus; *Thogotovirus*; Characterization; Molecular; Serological; *In vitro* growth

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1. Background

The genus *Thogotovirus* of the family *Orthomyxoviridae* comprises a small group of primarily tick-associated viruses that are globally distributed. In general, *Thogotoviruses* have a negative-sense, RNA genome of six segments and are distantly related to the archetype virus of the family, influenza virus. Until recently, only two members of the genus that are found in the Eastern Hemisphere, Thogoto and Dhori viruses, have been associated with human infection and disease [1,2]. Prior to 2014, only one *Thogotovirus*, Aransas Bay virus had been identified in the United States [3,4]. Aransas Bay virus has not been associated with human disease.

In June of 2014, a previously healthy adult male from Kansas died from complications related to an illness marked by fever, thrombocytopenia and leukopenia [5]. Prior to becoming unwell, the man reported finding an engorged tick on his shoulder. An isolate was derived from the blood of this patient during the course of diagnostic testing [5]. This isolate was subsequently identified as a novel orthomyxovirus of the genus *Thogotovirus* by partial-genomic high throughput sequencing (HTS) and microscopy [5]. The virus was named Bourbon virus (BRBV) after the patient's county of residence [5]. To the best of our knowledge, BRBV represents the first human pathogen of the genus *Thogotovirus* to be identified in the Western Hemisphere.

2. Objectives

A key aim of this study was to provide a comprehensive description of BRBV through basic molecular and serological analyses. In addition, it was our goal to assess possible host and vector range associations through the evaluation of BRBV growth in a variety of vertebrate and invertebrate cell lines. Furthermore, we sought to generate polyclonal sera for use in protein and serological analyses through the inoculation of CD-1 mice with BRBV.

3. Study design

3.1. BRBV isolation and purification

An isolate of BRBV was derived directly from 200 μl of a 1:10 dilution of patient blood that was taken on day nine post onset of illness and inoculated onto confluent Vero cells in a T25 flask. The inoculated flask was then incubated at 37 °C and reviewed for cytopathic effect daily. Substantial cytopathic effect was observed at day 3 post-inoculation. The supernatant from infected cells was then harvested for further evaluations. For protein analysis, BRBV was grown in multiple flasks of confluent Vero cells and purified on a glycerol tartrate gradient as described by others [6].

3.2. High throughput sequencing and analysis

BRBV RNA was extracted and purified from 0.1 mL of gradient-purified virus using the QIAGEN QIAamp kit (Qiagen) and resuspended in 0.1 mL of RNAse-free dH_20. HTS was conducted using the Ion Torrent PGM system (Life Technologies) and methods described elsewhere [7]. The 5′ and 3′ terminal ends of BRBV genomic segments were determined

using the Invitrogen 5′/3′ RACE System kits (Life Technologies) according to the manufacturer's protocol, followed by capillary sequencing on an ABI 3130 instrument (Life Technologies). Sequence data generated by all methods, comprising greater than 4.5 million reads and generated from repeated runs, were analyzed using the CLC Genomics Workbench 7.5.1 (CLCbio) and NGen 4 (DNAstar) software programs, employing both *de novo* and templated assembly parameters. The total approximated average genome coverage across all genomic segments is 1000X. ORF determination was conducted using the EditSeq function of the Lasergene 9 package (DNAstar), and basic analyses of both nucleotide and amino acid sequences were conducted using Mega version 5 software [8].

3.3. Real-time fluorescent probe based RT-PCR

Real-time primers and FAM-labeled probes were designed to detect the PB1 polymerase subunit (segment 2) and NP nucleoprotein (segment 5) ORFs using the Primer3Plus website at (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and newly derived BRBV sequence data (Table 1). Real-time assays were performed to confirm the presence of BRBV RNA in the isolate and purified BRBV preparation (derived as described above) using the QIAGEN QuantiTect Probe RT-PCR kit (Qiagen).

3.4. Phylogenetic analyses

Phylogenetic analyses were conducted on BRBV and diverse *Thogotoviruses* that are represented in GenBank. Alignments of the deduced amino acid sequences of all major *Thogotovirus* ORFs were generated using the Clustal W function of MEGA version 5 software [8–10]. Maximum parsimony and neighbor-joining (NJ) trees were generated and analyzed with 2000 replicates for bootstrap testing [10,8] with highly similar topologies generated by all methods. Generated NJ trees were chosen as representative of trees that were generated by all methods (Fig. 1).

3.5. Preparation of immune sera

CD-1 mice were inoculated with BRBV preparations ranging in titer from 0.1–1,000 PFU/0.1 mL inoculation using both intraperitoneal (IP) and intracranial (IC) routes of injection. All methods were conducted in accordance with institutional standards for the care and use of laboratory animals. After inoculation, mice were monitored daily for signs of morbidity and mortality. On day 24 post-inoculation (dpi), surviving mice [$n = 25$ (IP) and $n = 24$ (IC)] were bled by cardiac puncture. Resultant sera were screened by PRNT using a 90% plaque reduction threshold to confirm anti-BRBV activity and stored frozen at −80 °C for further analyses.

3.6. Immunoblotting

BRBV proteins from purified virus were separated by SDS-PAGE on a reduced 4–12% Bis/Tris gel (Invitrogen). All procedures were performed at room temperature. Proteins were blotted electrophoretically from the gels onto nitrocellulose membranes and washed for 15 min in PBS/0.1% Tween wash buffer. Non-specific binding sites were blocked with 10% goat serum/PBS for one hour while rocking. Pooled mouse immune serum, derived as described above, was diluted 1:500 in PBS and incubated with the membrane for 1 hour with

gentle rocking. Membranes were washed again in PBS/0.1% Tween wash buffer three times for 5 min each. Goat anti-mouse antibody conjugated to alkaline phosphatase (Jackson Immunoresearch) was diluted 1:200 in PBS and incubated on the membrane for 1 hour with gentle rocking. Membranes were washed as described above, and BCIP/NBT phosphatase substrate (KPL) was added to the membrane until a color change appeared. The reaction was stopped by the addition of dH_20.

3.7. Two-way plaque reduction neutralization assays

For PRNT analyses, approximately 100 PFU of BRBV or reference *Thogotovirus* strain was incubated with serial dilutions of BRBV mouse immune serum or reference antibodies using methods described by others [11]. Incubated virus/antibody mixtures were then inoculated onto Vero cells which were subsequently overlaid with agar and incubated at 37 °C using methods described elsewhere [11]. Plaques were counted on day 4 using a 90% plaque reduction threshold. Thogotovirus reference strains and antibodies were provided by the Arbovirus Diseases Branch of the Division of Vector-Borne Diseases, Centers for Disease Control and Prevention (CDC). Species-level identities of the reference strains were confirmed by serological analyses prior to this study.

3.8. Cell culture and BRBV growth kinetics

To evaluate growth kinetics in culture, a variety of vertebrate and invertebrate cell lines were inoculated in duplicate at approximately 0.1 MOI of BRBV in T25 flasks. These cell lines included kidney cells of monkey (Vero, Vero E6 and LLC-MK2) and hamster (BHK21Cl-15) origin, human hepatocarcinoma and cervical carcinoma (HUH-7 and HeLa), duck embryo (DE), frog (XLK-WG), *Aedes, Anopheles and Culex* spp. mosquito (CCL-125, C6/36, C7/10, AP-61, *Culex quinquefasciatus*) and *Dermacentor* (DAE15, [12]; ANE58, [13], *Amblyomma, Rh ipicephalus,* and *Hyalomma* (AVL/CTVM17, [14]; RAE/CTVM1, [14]; HAE/CTVM9, [15] spp. tick cells. Inoculated flasks were then incubated under optimal, cell line-specific conditions. Supernatants were collected from infected cells on a daily basis ranging from day 0 to day 15 and were subjected to plaque assay [11] to determine BRBV titers expressed as PFU/mL.

4. Results

4.1. HTS and phylogenetic analyses

Analyzed data from HTS reveal six RNA segments of the BRBV genome and the major proteins, generally shared among all *Thogotoviruses*, encoded therein (Table 2). Major ORFs include those that encode a putative PB2 polymerase subunit (segment 1), PBI polymerase subunit (segment 2), PA polymerase subunit (segment 3), GP glycoprotein (segment 4), NP nucleoprotein (segment 5), and M matrix protein (segment 6). When compared to the amino acid sequences of previously described *Thogotoviruses*, the translated ORFs of BRBV share percentage identities across all coding regions that range from ~24 to 82%. The percentage identities that BRBV shares with Dhori virus and its subtype, Batken virus, the two members of the genus with which BRBV shares the highest percentage identities, range from ~59% identity in the putative GP (most divergent) ORF to ~82% identity to in the putative PBI subunit (relatively conserved) ORF. Comprehensive

phylogenetic analyses (Fig. 1) generally support these findings, with BRBV grouping with strong support in all analyzed segments with Dhori and Batken viruses.

4.2. Evaluation of immune sera

Fifty five percent (27/49) serum samples collected from mice inoculated with BRBV were determined to possess specific anti-BRBV activity by PRNT analyses. This includes most mice (85%, 17/20) that were inoculated with relatively high titers of between 100 and 1000 PFU/inoculation of BRBV by both IC and IP routes. IP inoculations resulted in a higher proportion of mice generating anti-BRBV antibodies (76%, 19/25) than those receiving IC inoculation (33%, 8/24) ($P < 0.01$). Of interest, mice inoculated by both routes showed no signs of morbidity (i.e. tremors, sick rodent posture, ruffled fur, dehydration, extended hunched posture, recumbency, lack of response to stimuli, signs of paralyses or limb weakness, unresponsiveness) or mortality during the 24 day post-inoculation period.

4.3. Protein and serological analyses

Purified BRBV was separated as described to determine immunogenicity of the viral structural proteins. Pooled sera from mice inoculated with BRBV reacted with all three structural proteins: glycoprotein (GP) (approximately 60 KDa), nucleocapsid (NP) protein (approximately 52 KDa), and matrix (M) protein (approximately 30 KDa; Fig. 2). These results generally confirm the masses calculated from the translated putative structural protein ORFs represented in Table 2.

Results from 2-way PRNT analyses reveal that for all represented *Thogotoviruses*, a high degree (≥160 PRNT titer) of neutralizing activity is only seen with mouse immune serum or reference antibodies for the specific virus strain against which they were generated (Table 3). These findings indicate a low level of serological cross-reactivity among *Thogotoviruses*. Low levels of one-way cross-neutralization were observed for BRBV with anti-Dhori virus mouse serum (PRNT titer of 20; Table 3), which supports the phylogenetic determination of a degree of distant relatedness between these two viruses (Fig. 1).

4.4. Virus growth kinetics

Comprehensive analyses of the growth kinetics of BRBV are presented (Fig. 3). Of note, the highest titers of between ~$10^{\hat{7}}$ and $10^{\hat{9}}$ PFU/mL of BRBV are documented in supernatants taken from hamster (CHK-Cl-15) and monkey (Vero) cell lines over the 15 day time course (Fig. 3). Also, sustained growth of BRBV in several tick cell lines is documented (Fig. 3). Specifically, BRBV was shown to replicate to high titers of ~$10^{\hat{5}}$ and $10^{\hat{7}}$ PFU/mL in *Hyalomma* and *Rhipicephalus* cell lines (RAE/CTVM1 and HAE/CTVM9) with intermediate titers of ~$10^{\hat{4}}$ –$10^{\hat{6}}$ PFU/mL achieved in the *Amblyomma* cell line, AVL/CTVM17 over the 14 day time course (Fig. 3). Less robust growth of ~$10^{\hat{1}}$ –$10^{\hat{5}}$ PFU/.1 mL was observed in the *Dermacentor* cell lines, DAE15 and ANE58 (Fig. 3). Finally, weak and declining BRBV titers were derived from all evaluated mosquito cell lines (Fig. 3).

5. Discussion

To support both research and diagnostic aims, we have conducted basic analyses of BRBV at the molecular and serological levels. Findings from these analyses definitively identify BRBV as a distinct member of the genus *Thogotovirus*. Phylogenetically, BRBV is most closely related to Dhori virus and its subtype, Batken virus (Fig. 1) that together have been known to occur in regions throughout Africa, Asia and Europe [16–18,1,19]. Dhori and Batken viruses have been isolated from *Hyalomma* ticks and antibodies to Dhori virus have been identified in camels, goats, horses, cattle and humans [16–18,1,19]. Of interest, Batken virus has also been isolated from several mosquito species [19].

Robust growth in a variety of invertebrate and vertebrate cell lines suggests a probable association between BRBV and mammalian and tick hosts (Fig. 3). In addition, relatively high titers derived from all mammalian cells are compatible with the known susceptibility of the human host to BRBV infection and disease (Fig. 3). Of note, the high titers of BRBV derived over a sustained time course from hamster cells is possibly reflective of a small mammal serving as an amplification host (Fig. 3). Certainly, the susceptibility of CD-1 mice to BRBV infection as represented by seroconversion in the absence of morbidity and mortality (see Results) is consistent with this hypothesis. As an aside, the lack of morbidity and mortality in BRBV-infected CD-1 mice indicates that they are unlikely to be useful in the development of a murine model for BRBV-related human disease. Although, more comprehensive investigations, including analyses of viral load in infected mice, are recommended to better understand the replication and pathology of BRBV in the murine host.

Growth of BRBV in cell lines derived from the hard ticks *Hyalomma*, *Rhipicephalus* and *Amblyomma* suggests a likely association of BRBV with a tick vector (Fig. 3). Furthermore, the geographic location of the BRBV infection discussed here is coincident with geographic distribution of a number of tick species, including *Amblyomma americanum* that has been associated with the human pathogens *Ehrlichia chaffeensis* and Heartland virus [20,21]. Although, differential speculation on species-level tick host associations is not made at this time and it is noted that no soft tick cell lines were included in this study. Of interest, Thogoto virus, the type species of the genus *Thogotovirus*, also grows in the HAE/CTVM9 and RAE/CTVM1 cell lines used in the present study [22]. Finally, weak and declining titers of BRBV derived from mosquito cell lines are reflective of a lack of replication and an unlikely association between BRBV and a mosquito vector (Fig. 3).

While not discussed here, BRBV was discovered as part of an epidemiological study of the relatively newly discovered, tick-associated Heartland virus [4]. The isolation of BRBV as part of the diagnostic component of this study highlights the probability that there are other, as of yet undiscovered, tick-associated pathogens within the United States and elsewhere. Taken together, the data presented herein suggest a relatively distant ancestral divergence of BRBV from other *Thogotoviruses*. Such divergence is suggestive of the long-term wide dispersal of *Thogotoviruses* globally and the plausible isolated evolution of BRBV in the Western Hemisphere. Furthermore, these findings are consistent with the hypothesis that BRBV has been circulating in the United States, undetected, for many years. Finally, it is

important to note that while an association between BRBV and a tick vector has been suggested through clinical history [4], phylogenetics and *in vitro* characterization, the host associations and transmission dynamics of BRBV have yet to be conclusively determined through more comprehensive investigations.

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Abbreviations

HTS	high throughput sequencing
BRBV	Bourbon virus
CDS	Centers for Disease Control and Prevention
RT-PCR	reverse transcription polymerase chain reaction
ORF	open reading frame
NJ	neighbor joining
PFU	plaque forming unit
IP	intraperitoneal
IC	intracranial
DPI	days post onset
PRNT	plaque reduction neutralization test
MOI	multiplicity of infection

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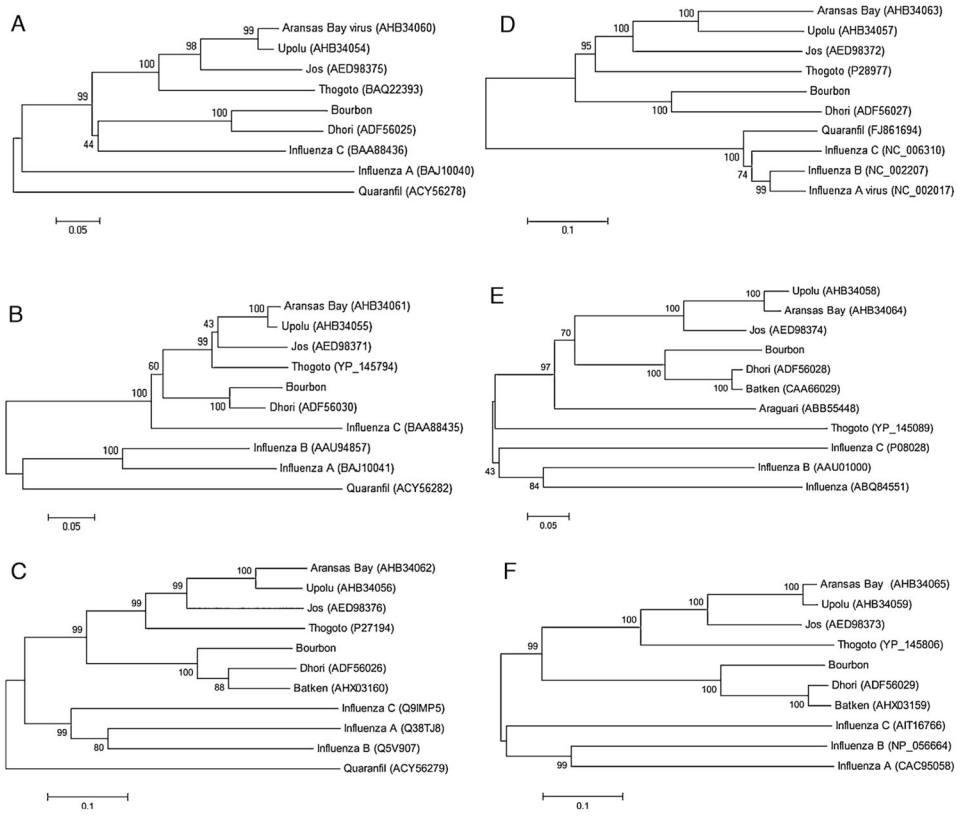

Fig. 1.

Phylogenies of deduced amino acid sequences of representative genes of Bourbon virus in comparison to homologous sequences of selected orthomyxoviruses. A neighbor-joining method was used in the inference of each phylogeny with 2000 replicates for bootstrap testing. (A) PB2, (segment 1) (B) PB1, (segment 2) (C) PA, (segment 3) (D) GP, (segment 4), (E) NP, (segment 5) (F) M, (segment 6). Scale bars represent the number of amino acid substitutions per site. GenBank accession numbers appear next to taxon names. Phylogenies based upon partial PA, NP and M sequences also appear elsewhere [4]. Phylogenetic comparisons including Batken virus were not made for all segments as only partial data are available for this virus in GenBank.

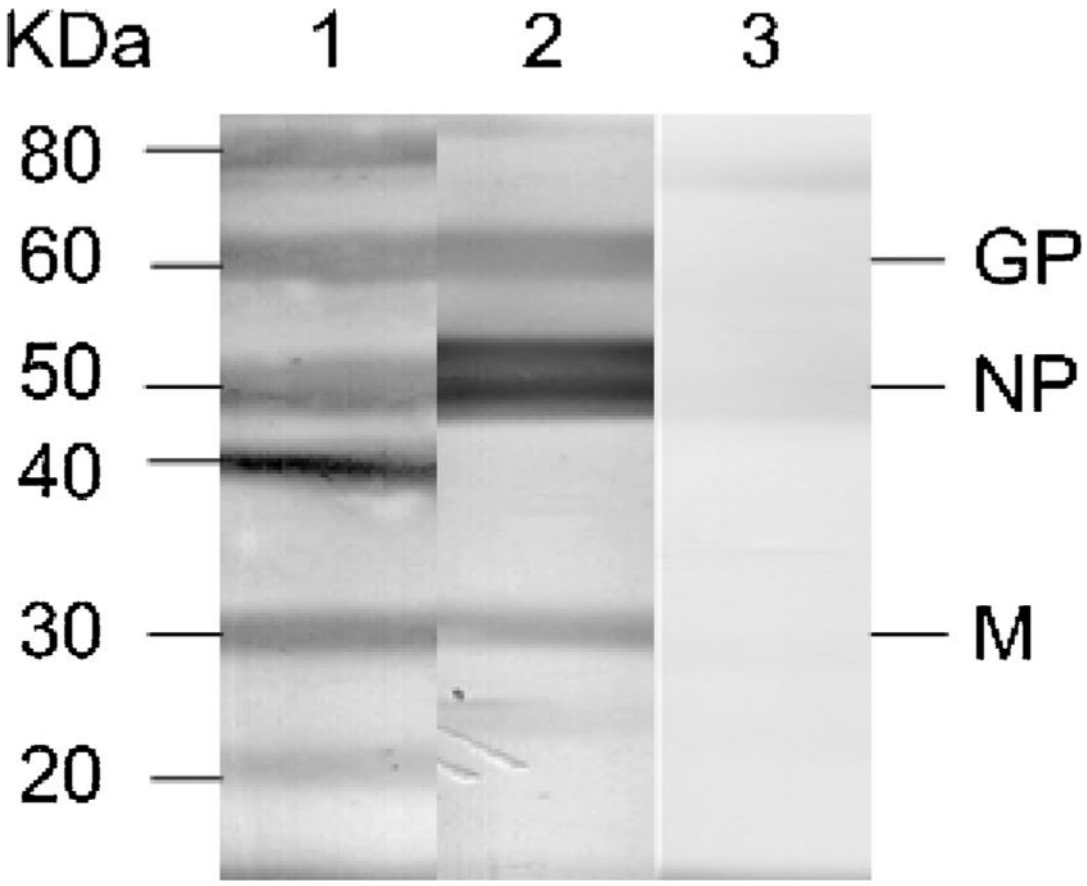

Fig. 2.

Protein specificities of antibodies elicited to BRBV structural proteins. A Western blot of BRBV proteins was prepared as described in the Materials and Methods section. Lane 1, Novex Sharp pre-stained protein standard (sizes are shown in KDa); Lane 2, pooled mouse sera after infection with 10^3 and 10^2 PFU BRBV inoculated intraperitoneally; Lane 3, pooled mouse sera from uninfected mice. Estimated BRBV GP, glycoprotein, NP, nucleoprotein, and M, matrix protein sizes are indicated.

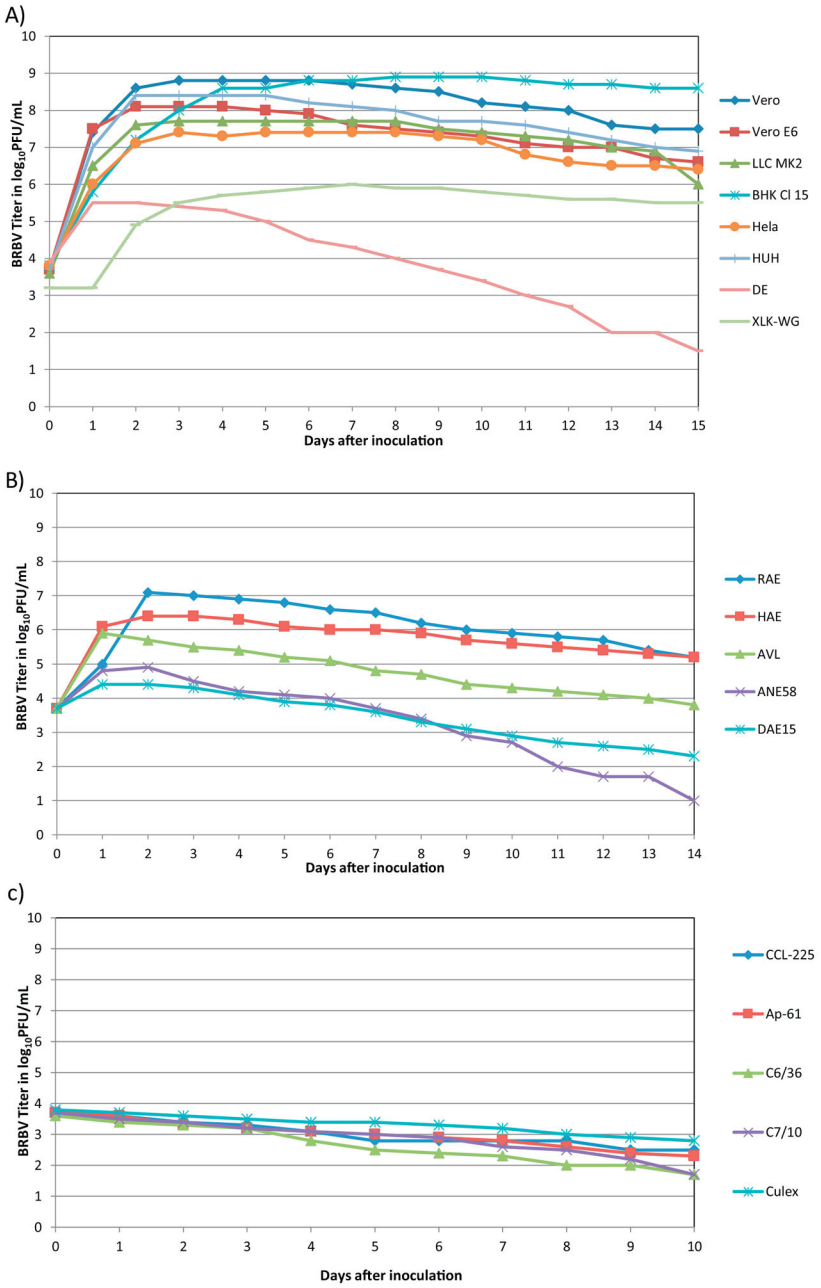

Fig. 3.

Comparative study of Bourbon virus growth kinetics in 18 cell lines. Growth kinetics are presented for (A) eight vertebrate cell lines: Vero, Vero E6, LLC MK2, BHK Cl 15, Hela, HUH-7, DE and XLK-WG, (B) five tick cell lines: DAE15, ANE58, AVL/CTVM17 (AVL), RAE/CTVM1 (RAE) and HAE/CTVM9 (HAE) and (c) five mosquito cell lines: *Aedes aegypti* (CCL-225), C6/36, C7/10, AP-61, and *Culex quinquefasciatus* (Culex). Virus titers determined by plaque assay in Vero cells.

Table 1

Real-time primers for the detection of Bourbon virus RNA.

Segment	Name[a]	Position[b]	Sequence
2	PBI1F	742–761	AACCGAAGGACCATTGCTAC
2	PBI1R	832–851	ACAGGGACTCCAGAACTTGG
2	PBIprobe	794–815	ACCCTTGCTGCATCTTCCACCA
5	NPI1F	1150–1169	GCAAGAAGAGGCCAGATTTC
5	NPI1R	1276–1295	TCGAATTCAGCATTCAGAGC
5	NPprobe	1177–1197	CCTCACACCACGGAAGCTGGG

[a] Probe designation indicates 5′FAM labeled probe.

[b] Positions given in coding, plus sense.

Table 2

Summary of Bourbon virus genomic organization, major ORF and putative proteins.

Segment	Length (nt)	ORF size	Putative Protein	Predicted MW-KD	Range of Amino Acid Percent identity W/Known *Thogoviruses*[a]	Amino acid Identity W/Dhori virus
1	2726	2349	PB2	89	35–74%	74%
2	2200	2148	PB1	81	60–82%	82%
3	1961	1911	PA	73	36k67%	67%
4	1588	1539	GP	58	35–59%	59%
5	1480	1383	NP	52	44–70%	70%
6	960	813	M	31	24–74%	74%

[a]Compared *Thogotoviruses* include those for which there are full-length genomic sequences in GenBank.

Table 3

Thogotovirus cross-reactivity as determined by plaque reduction neutralization.

	Viruses				
Polyclonal antibody source	**BRBV**	**DHOV**	**THOV**	**ARAV**	**UPOV**
Bourbon virus (**BRBV**) mouse serum	*5120*[a]	<10	<10	<10	<10
Dhori (**DHOV**) MHIAF[b]	20	≥*20480*	<10	<10	<10
Thogoto (**THOV**) MHIAF	<10	<10	160	<10	<10
Aransas bay (**ARAV**) virus MHIAF	<10	<10	<10	*2560*	<10
Upolu (**UPOV**) MHIAF	<10	<10	<10	<10	≥*20480*

[a]Values represent the reciprocal of the dilution of serum at which 90% plaque reduction was achieved. Homologous titers are italicized.

[b]Mouse hyperimmune ascites fluid (MHIAF).