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Experimental infection of big brown bats (*Eptesicus fuscus*) with Eurasian bat lyssaviruses Aravan, Khujand, and Irkut virus

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Summary. Here we describe the results of experimental infections of captive big brown bats (*Eptesicus fuscus*) with three newly isolated bat lyssaviruses from Eurasia (Aravan, Khujand, and Irkut viruses). Infection of *E. fuscus* was moderate (total, 55–75%). There was no evidence of transmission to in-contact cage mates. Incubation periods for Irkut virus infection were significantly shorter ($p < 0.05$) than for either Aravan or Khujand virus infections. In turn, quantification of viral RNA by TaqMan PCR suggests that the dynamics of Irkut virus infection may differ from those of Aravan/Khujand virus infection. Although infectious virus and viral RNA were detected in the brain of every rabid animal, dissemination to non-neuronal tissues was limited. Levels of viral RNA in brain of Aravan/Khujand virus-infected bats was significantly correlated with the number of other tissues positive by TaqMan PCR ($p < 0.05$), whereas no such relationship was observed for Irkut virus infection (where viral RNA was consistently detected in all tissues other than kidney). Infectious virus was isolated sporadically from salivary glands, and both infectious virus and viral RNA were obtained from oral swabs. The detection of viral RNA in oral swabs suggests that viral shedding in saliva occurred <5 days before the onset of clinical disease.

Introduction

The majority of lyssaviruses described to date are associated with infection of bats. In the Americas, this is restricted to different phylogenetic lineages of rabies virus (RABV; genotype 1) [20]. Lyssavirus infection of Old World bats is caused by a number of different genotypes: Lagos bat virus (LBV; genotype 2), Duvenhage virus (DUVV; genotype 4), European bat lyssaviruses types 1 and 2 (EBLV-1 and EBLV-2; genotypes 5 and 6, respectively), Australian bat lyssavirus

(ABLV; genotype 7) [1]. The recent discovery of the Eurasian bat lyssaviruses Aravan, Khujand [16], Irkut, and West Caucasian bat virus [5] may require a reassessment of lyssavirus taxonomy as each virus has been proposed as a putative new genotype [17].

Most experimental studies on the pathogenesis and transmission of bat rabies have been performed using RABV [2, 8, 19, 22–25]. Pathogenesis and transmission of non-rabies lyssaviruses has not been studied sufficiently, in part due to protection of European bat species. In the two published studies of experimental infection of bats with non-RABV lyssaviruses (EBLV-1 and Aravan virus), rather traditional patterns of bat rabies were described [4, 15]. Infection resulted in fatal encephalitis; virus was found in the brain of all dying bats, and frequently in their salivary glands [4, 15]. In a small number of rabid animals infected with EBLV-1 and Aravan virus, low titres of virus have been detected in non-neuronal tissues (brown fat, lungs, kidneys). Survival from clinical rabies has not been described. Little to nothing is known regarding the extraneural tissue distribution of virus in naturally-infected bats [3, 7], but the dynamics of pathogenesis should not differ drastically from that of classical RABV infection.

Limited evidence suggests that maintenance and transmission of lyssaviruses within bat populations may include a low level of abortive infection. Field studies in Spanish bat populations have demonstrated the presence of EBLV-1 RNA in oral swabs of apparently healthy bats [11] which, coupled with the long-term dynamics of anti-EBLV-1 antibodies [21], suggests that bats may under certain circumstances survive infection. Similarly, longitudinal serological surveillance of a captive colony of *E. fuscus* suggests that virus-neutralizing antibody induction may be indicative of a protective immune response rather than pathogen incubation and subsequent development of an infectious state [23]. A potentially analogous situation may exist for non-RABV bat lyssaviruses.

In the present work, we have performed experimental infections of *E. fuscus* with Irkut, Aravan, and Khujand viruses. Viral pathogenesis was assessed using measures of viral RNA and infectious virus detection in a variety of tissues. Besides providing valuable insights into the pathogenesis of lyssaviruses, the results of this study served to assess the suitability of *E. fuscus* as a model for infection of other bat species.

Materials and methods

Animals

Adult *E. fuscus* of both sexes were captured from two roosts in suburban areas of Georgia, USA. This region is known to be endemic for bat rabies, but no recent bat carcasses or rabid bats were found in or near the vicinity of the roosts. In order to reduce stress during the period of adaptation to captivity, pre-experimental tests for detection of anti-RABV antibodies were not performed. Animals were individually tagged and housed in metal cages, three bats per cage. Four to five weeks of quarantine were allowed for adaptation to captivity. No rabid bats were detected during this period of adaptation to captivity. All animal care and experimental procedures were performed in compliance with the Centers for Disease Control and Prevention Institutional Animal Care and Use Guidelines.

Viruses

Irkut, Aravan, and Khujand viruses were used as inocula after 2 to 3 intracerebral passages in mice. Ten-percent mouse brain suspensions were prepared in Minimum Essential Medium (MEM-10; GIBCO, Invitrogen Corp., Gaithersburg, MD, USA) supplemented with 10% fetal calf serum and clarified by centrifugation at 3200 *g* for 15 min. Aliquots of supernatant were stored at −80 °C and used for experimental inoculations and titration in mice to confirm dose. Inoculation doses used were 3.9 $\log_{10}$ 50% mouse intracerebral lethal doses ($MICLD_{50}$) for Aravan virus, 4.3 $\log_{10}$ $MICLD_{50}$ for Khujand virus, and 4.7 $\log_{10}$ $MICLD_{50}$ for Irkut virus.

Experimental infections and sampling

In each cage, one or two bats were inoculated intramuscularly in the neck or deltoid muscle with 50 µl of Irkut, Aravan, or Khujand virus suspensions (Table 1). Non-inoculated cage mates (one or two per cage) were kept in-contact for the duration of the experiment.

Oral swabs were taken weekly during the observation period prior to the experiment, and twice a week for 3 to 5 weeks post-inoculation. At each time point, two swabs were taken from each bat (using sterile cotton tipped swabs): one swab was placed directly into 1 ml of MEM-10 for virus isolation, the other into 1 ml of TRIzol reagent (GIBCO-BRL, Inc., Gaithersburg, MD, USA) for RNA extraction. Swab samples were placed immediately on dry ice and stored at −80 °C. Where possible, swabs were taken from all bats.

Whenever bats were found dead, necropsy was performed as quickly as possible. When animals were observed in the terminal stage of disease, euthanasia was performed using 50 µl of Ketaset III (100 mg/ml; Fort Dodge Animal Health, Fort Dodge, Iowa, USA) and necropsy performed shortly after. Tissue samples from brain, salivary glands (submandibular and parotid), brown fat, lung, kidney, tongue (Aravan and Khujand virus-infected bats only), spleen (Aravan and Khujand virus-infected bats only), and bladder (Irkut virus-infected bats only) were removed at necropsy and stored at −80 °C.

All surviving bats from the Aravan and Khujand virus experiments (both inoculated and in-contact) were euthanized six months after the inoculation date. All surviving bats (both inoculated and in-contact) from the Irkut virus experiment were euthanized approximately one year after the inoculation date. Only brain and salivary gland samples were taken from surviving bats at post-mortem.

Direct fluorescent antibody test (FAT)

The FAT was performed according to standard recommendations [27] using fluorescein-isothiocyanate-conjugated anti-RABV monoclonal immunoglobulin (Centocor Inc., Malvern, PA, USA). All brain samples (i.e., those from experimental bats and those from mice used for MIT and virus titrations) and infected cell cultures were tested by FAT.

Rapid tissue culture infection test (RTCIT)

RTCIT was performed as previously described [6] with a slight modification. Ten-percent suspensions of bat tissues were prepared using MEM-10 and centrifuged at 4000 *g* for 10 min. Supernatant was then used for virus isolation and mouse inoculation test, and the sediment used for RNA extraction (see below).

For the RTCIT, 300 µl of a mouse neuroblastoma cell suspension (MNA; CDC, Atlanta, GA, USA) in MEM-10 (final concentration $\sim 2 \times 10^5$ cells/ml) were placed into each well of an eight-chamber LabTek slide (Nalge Nunc International, Naperville, IL, USA). One hundred microlitres of tissue supernatant were then added to individual wells on two slides and

Table 1. Experimental details, course and outcome of lyssavirus infections

Virus	Bat ID#	Inoculated or in-contact	Route of inoculation	Incubation period (days)	Clinical period (days)	Outcome
Khujand	8	I	N	19	2	F
	23	I	N	14	1	E
	25	I	D	20	2	E
	37	I	D	–	–	S
	40	I	D	–	–	S
	24	C	–	–	–	S
	6	C	–	–	–	S
	14	C	–	–	–	S
Aravan	7	I	D	22	2	E
	38	I	D	16	2	E
	39	I	N	20	2	E
	43	I	D	–	–	S
	12	C	–	–	–	S
	21	C	–	–	–	S
	35	C	–	–	–	S
Irkut	891	I	D	12	1	E
	893	I	D	12	2	E
	909	I	D	14	2	F
	910	I	D	7	1	E
	911	I	D	16	2	E
	917	I	D	7	2	E
	883	I	D	–	–	S
	887	I	D	–	–	S
	884	I	D	–	–	S
	877	I	D	–	–	S
	921	I	D	–	–	S
	890	C	–	–	–	S
	919	C	–	–	–	S
	900	C	–	–	–	S
	920	C	–	–	–	S
	912	C	–	–	–	S
	922	C	–	–	–	S
	915	C	–	–	–	S

I inoculated, *C* in-contact, *N* neck, *D* deltoid muscle, *F* fatal infection, *E* euthanasia, *S* survival

mixed with the cell suspension. Chambers were closed and incubated in a CO_2 incubator (Forma Scientific Inc., Marietta, OH, USA) at 37 °C. One set of slides was tested by FAT at 48 h post-inoculation of cells, and the second set tested at 72 h post-inoculation of cells. If the 10% tissue suspension was cytotoxic, a further 10-fold dilution was tested by RTCIT.

In the same manner, RTCIT was also used for oral swabs which had been agitated in MEM-10. Tubes containing oral swabs were thawed, and the swabs, after being slightly wrung on the tube wall, were discarded. One hundred µl of swab medium was then tested in the RTCIT.

Oral swabs positive for viral RNA (see below), and swabs taken from the same animals one week prior to the first RNA-positive swab, were additionally subjected to three 72-hour

passages in inoculated cells in 25-cm^2 flasks (Corning, NY, USA). Results of each cell passage were tested by FAT at 48 and 72 h post-inoculation.

Mouse inoculation test (MIT) and virus titration

Intracerebral MIT was performed for both tissue homogenates of salivary gland (10% suspension in MEM-10) and 10-fold dilutions of the inocula, as described elsewhere [27] using 3-week old inbred mice. Viral titres were calculated using the Spearman-Karber method [27].

TaqMan polymerase chain reaction (TaqMan PCR)

Total RNA was extracted from both the sediment of 10% bat tissue suspensions (200 µl per extraction) and oral swabs (which had been placed directly into TRIzol) using TRIzol reagent according to the manufacturer's instructions. For TaqMan PCR, 1 µl of RNA was reverse transcribed using Reverse Transcription System (Promega Corp., Madison, WI, USA) with 0.5 µg of the supplied random hexamers. TaqMan PCR was performed as described elsewhere but without normalization according to β-actin mRNA levels [13]. TaqMan PCR was performed using the primers and probes listed in Table 2, all of which were designed using the Primer Express software (Applied Biosystems, Foster City, California). Real-time PCR determines levels of sequence-specific nucleic acid using the accumulation of a fluorescent dye (attached to a sequence-specific probe), which is released during the PCR. The thermocycle

Table 2. Oligonucleotide primers and probes used for PCR-based methods of lyssavirus detection

Target virus	Role	Sequence (5′ ⟶ 3′)	Position
Aravan	F	AGTATCCTGCCATTGGGAATCA	86–107
	R	GACTGTTGTAACTGCATATGAG	681–702
	NF	ATGACGTCTGTTCTTATCTTGCT	203–225
	NR	ATGTGTGCAAATTGGAGCACG	577–597
	TaqMan F	CTTCGTCAAGGTGGTTGAACATC	531–553
	TaqMan R	TGGAGCACGATACCAAATTTCA	589–610
	TaqMan P	CATTGATGACCACTCACAAGATGTGTGCAA	557–586
Khujand	F	AATACCCGGCTATTGTGGATAG	86–107
	R	GAACTGTTGTGACCGCATATGA	680–701
	NF	ATGATGTCTGTTCTTATTTGGCC	203–225
	NR	AATGTGTGCTAACTGGAGCACA	576–597
	TaqMan F	AACTGGGCATTGACCGGAG	355–373
	TaqMan R	ACATGCATCCTTAGTGGGGCT	408–428
	TaqMan P	CTAGACCTGACAAGAGATCCGACTGTAGCCG	376–406
Irkut	F	AATACCCTGCGATTGATGATAA	86–107
	R	GAACAGTTGTGACTGCATATGA	680–701
	NF	ATGATGTGTGCTCTTACTTGGCT	203–225
	NR	AATGTGTGCAAATTGGAGCACA	576–597
	TaqMan F	GTAATTGGGCTCAGGCAGGAG	353–373
	TaqMan R	AGGAGCCCGACTAAAGACGC	412–431
	TaqMan P	ACAAGACCTTACTAGAGATCCAACAACACCGGAAC	375–409

F forward (and RT) PCR primer, *R* reverse PCR primer, *NF* nested forward PCR primer, *NR* nested reverse PCR primer, *P* TaqMan PCR probe, Position: relative to the Pasteur virus nucleoprotein gene (GenBank accession number M13215)

number at which the fluorescence of the reaction rises above a threshold value (threshold cycle number; C_t) is used as a measure of the levels of target nucleic acid within the sample. TaqMan PCR was performed for 40 thermocycles and TaqMan PCR quantities calculated as 40 minus the mean C_t of three replicate reactions. TaqMan PCR was performed on RNA extracted from tissues and oral swabs from all bats.

Reverse transcription polymerase chain reaction (RT-PCR) and nested RT- PCR (nRT-PCR)

For RT-PCR and nRT-PCR, cDNA was generated by reverse transcription of RNA with a forward primer (Table 2) for 60 min at 42 °C in the presence of dNTPs and RT AMV (Roche Diagnostics Corp., Mannheim, Germany). PCR was performed for 40 thermocycles (94 °C for 30", 37 °C for 30", 72 °C for 90"; final extension 70 °C for 7′) in the presence of both the sense and anti-sense primers (Table 2) and *Taq* DNA polymerase (Roche Molecular Systems, Inc., Branchburg, NJ, USA). PCR products were visualized by gel electrophoresis using a 2% agarose gel stained with ethidium bromide (0.5 μg/ml). For nRT-PCR, primary PCR products were diluted 10-fold with water and subjected to 40 identical cycles of PCR using nested primers (Table 2). Positive (RNA from mouse brain infected with each lyssavirus) and negative control (water) samples were used with each PCR run. The RT-PCR and nRT-PCR were only performed on tissues from bats inoculated with Irkut virus. Oral swabs taken during all three experiments were tested by both RT-PCR and nRT-PCR.

Nucleotide sequencing

Nucleotide sequencing was performed on all nRT-PCR products derived from oral swabs and tissue samples. The PCR products were purified using Wizard PCR Preps DNA Purification Systems (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Purified product was sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA, USA) with the nested forward primer as in the manufacturer's instructions. Sequencing reactions were purified using Centrisep Spin Columns (Princeton Separations, Adelphia, NJ, USA), run on an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were compared to sequences of the relevant virus (GenBank accession numbers AY262023, AY262024, and AY333112 for Aravan, Khujand, and Irkut viruses, respectively).

Statistical analysis

All statistical testing was carried out using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Disease progression and clinical observations

The details of infection for bats from each experiment are shown in Table 1. Incubation periods following inoculation with Irkut virus (mean = 11.3 days; 95% confidence limits [CL] 8.3–14.3) were significantly shorter than those following inoculation with both Aravan virus (mean = 19.3 days; 95% CL 22.9–15.8; Mann Whitney U test, $p = 0.026$) and Khujand virus (mean = 18.0 days; 95% CL 21.7–14.3; Mann Whitney U test, $p = 0.048$).

Three of four bats inoculated with Aravan virus developed clinical signs compatible with rabies. One bat exhibited agitation, aggressiveness, and tremors followed by general weakness and lethargy. Two others demonstrated only weakness and lethargy for 1–2 days, and were subsequently euthanized.

For Khujand virus infection, disease progression was rapid; between 1 and 4 days, with clinical signs becoming more severe with time. Signs included weakness, ataxia, and paralysis; aggression was observed for only one animal. Two bats were euthanized during the lethargic stage, and one was found dead at the cage bottom.

Six of eleven bats inoculated with Irkut virus developed clinical disease; five were euthanized upon the appearance of lethargy, and one bat was found dead.

Virus isolation

The results of laboratory testing for infectious virus in tissue samples are presented in Figs. 1 and 2. Infectious virus was found in brain of every rabid animal. Virus was isolated inconsistently from salivary gland: from five bats (83.3%) that succumbed to Irkut virus (titres 2.3–3.1 $\log_{10}$ $MICLD_{50}$/0.03 ml) and one bat (33.3%) that succumbed to Khujand virus (titre 2.1 $MICLD_{50}$/0.03 ml). Virus was also isolated from lung tissue from two bats (33.3%) that succumbed to Irkut virus and one bat (33.3%) that succumbed to Khujand virus, from brown fat of 2 bats (66.6%) that succumbed to Khujand virus, and from tongue of 1 bat (33.3%) that succumbed to Khujand virus. Virus titres in these tissues were not determined. For bats that succumbed to Aravan virus, infectious virus could not be isolated from any tissue other than brain.

PCR-based methods for detection of viral RNA in tissue samples

As expected, viral RNA was detected more readily than infectious virus, especially for bats that succumbed to Irkut virus. Notably, the least number of positive results were also obtained from Aravan virus-infected bats. Brain and salivary gland of bats which survived inoculation for all three experiments, as well as those of in-contact bats, were negative for viral RNA.

TaqMan PCR quantities from tissues of bats are shown in Figs. 1 and 2. TaqMan PCR quantities were not normalized according to an endogenous control, so minor fluctuations may be present in the levels of RNA used for reverse transcription and subsequent TaqMan PCR. Nonetheless, the standardization of the RNA extraction method (i.e., amount of tissue used) minimizes inter-sample variation such that normalization does not drastically affect relative magnitudes of TaqMan PCR quantities (unpublished data). Logistic regression analysis using only the results from tissues positive by TaqMan PCR (combined for all three experiments; $n = 59$) shows a significant association between TaqMan PCR quantities and the recovery of infectious virus by RTCIT (Wald statistic $= 12.00$, $p = 0.001$).

Levels of viral RNA in tissues, determined by TaqMan PCR, show a markedly different profile for bats infected with Aravan/Khujand viruses and those infected

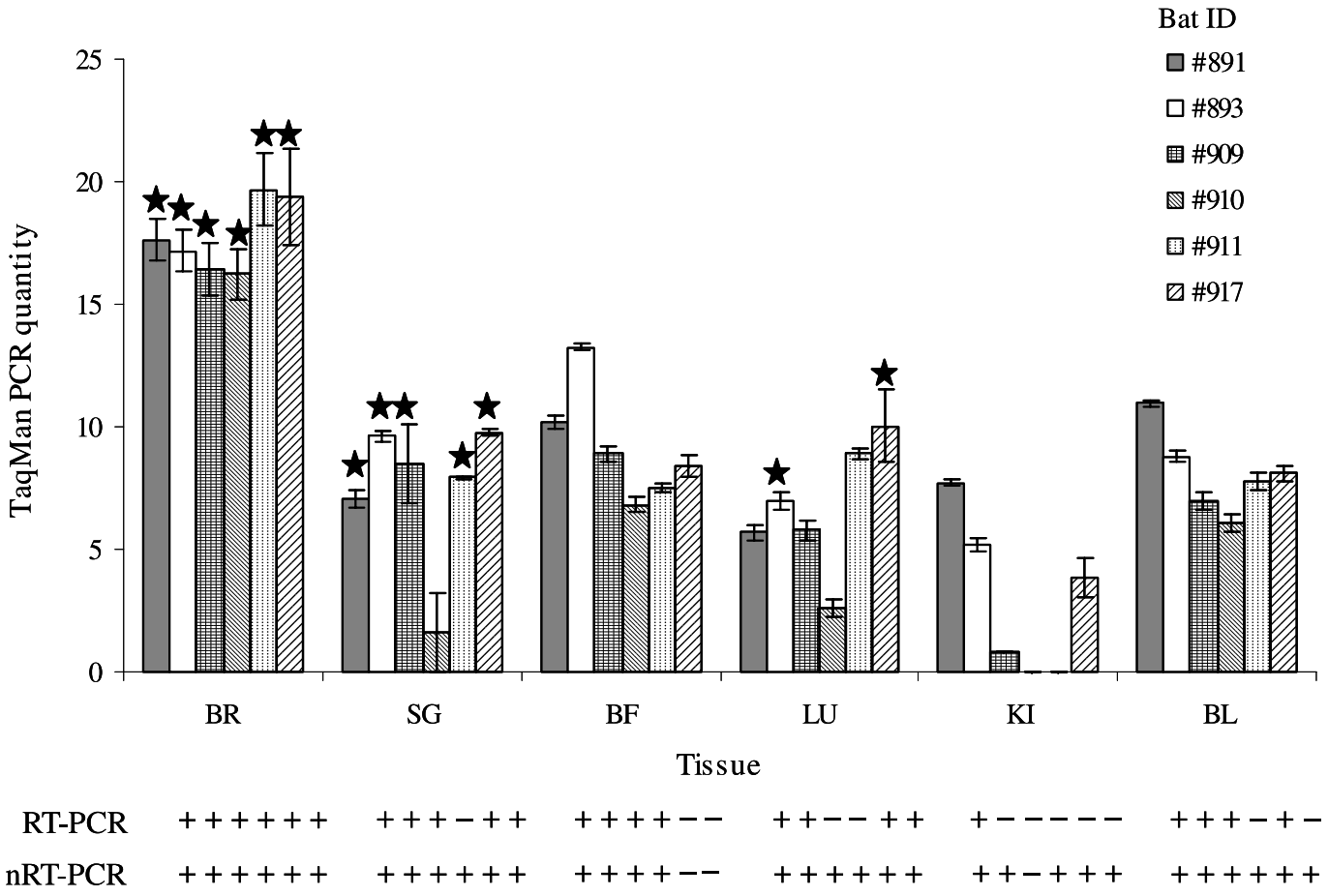

Fig. 1. TaqMan PCR quantities, results of RT-PCR and nRT-PCR, and results of virus isolation from tissue samples taken from bats experimentally infected with Irkut virus. Stars indicate tissue samples positive by virus isolation using the RTCIT. Error bars show 95% confidence limits of the mean from three replicate TaqMan PCRs. *BR* brain, *SG* submandibular and parotid salivary glands, *BF* brown fat, *LU* lung, *KI* kidney, *BL* bladder, + positive, − negative

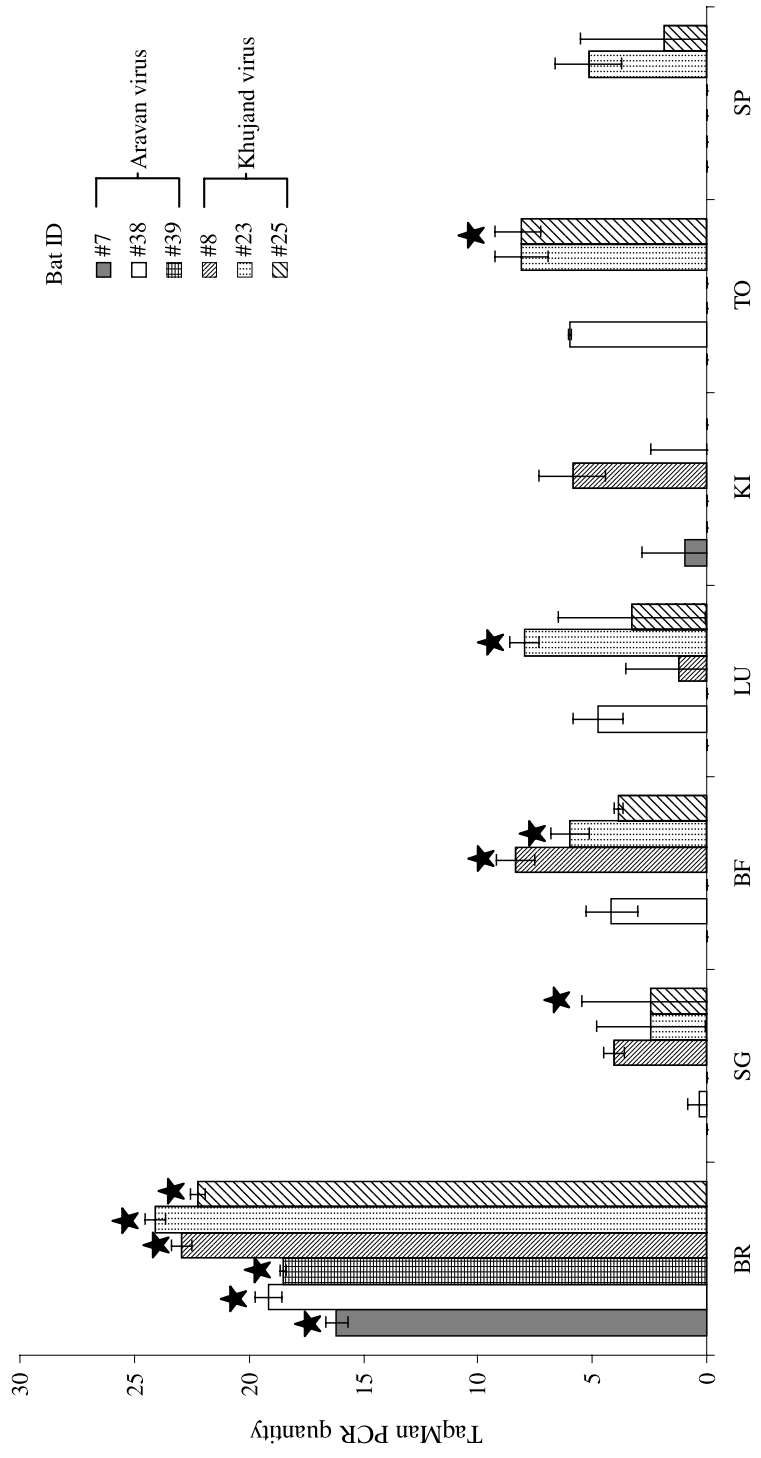

Fig. 2. TaqMan PCR quantities and results of virus isolation from tissue samples taken from bats experimentally infected with Aravan and Khujand virus. Stars indicate tissue samples positive by virus isolation using the RTCIT. Error bars show 95% confidence limits of the mean from three replicate TaqMan PCRs. *BR* brain, *SG* submandibular and parotid salivary glands, *BF* brown fat, *LU* lung, *KI* kidney, *TO* tongue, *SP* spleen, + positive, − negative

with Irkut virus (Figs. 1 and 2). Viral RNA was consistently detected in tissues of bats infected with Irkut virus, whereas levels of viral RNA in non-neuronal tissues of bats infected with Aravan or Khujand viruses were sporadic. The extent of viral RNA dissemination to non-neuronal tissues of bats infected with Aravan and Khujand viruses was related to the level of viral RNA in brain (Spearman's rank correlation coefficient $r_s = 0.88$, $n = 6$, $p = 0.02$), whereas no such relationship was observed for viral RNA levels from bats infected with Irkut virus ($r_s < 0.01$, $n = 6$, $p = 1.00$) and other combinations of data (Aravan and Irkut: $r_s = 0.105$, $n = 9$, $p = 0.787$; Khujand and Irkut: $r_s = 0.261$, $n = 9$, $p = 0.498$).

PCR-based methods for detection of viral RNA in oral swabs

To determine viral shedding during the experiments, oral swabs were first processed by PCR-based methods (Table 3). The RT-PCR detected one positive swab from bat #911 (infected with Irkut virus), taken on the day of euthanasia. TaqMan PCR detected five positive swabs (including the swab positive by conventional RT-PCR). The nRT-PCR detected seven positive swabs (including the five positive by TaqMan PCR). All positive swabs were obtained 0–2 days prior to the onset of clinical signs (or 0–4 days before euthanasia).

All oral swabs were negative by RTCIT. However, after the second cell culture passage, from swabs which were positive for viral RNA, two samples were positive by virus isolation. These were swabs from bat #911 (infected with Irkut virus) and bat #25 (infected with Khujand virus), both taken on the day of euthanasia. The third passage did not produce additional virus isolates. All other oral swabs,

Table 3. Results of PCR-based methods on oral swabs taken from lyssavirus-infected bats

		Day post inoculation										
Virus	Bat ID#	0	7	8	9	13	14	16	18	20	22	24
Aravan	7	–	–				–		–		–	–E
	38	–	–				–		–E			
	39	–	–				–		–	–	–E	
Khujand	8	–	–				–		$+^{TN}$	$+^{TN}$	D	
	23	–	–				E					
	25	–	–				–		–	$+^{TN}$	$+^{TN}$E	
Irkut	891	–	–			E						
	893	–	–				$+^{N}$E					
	909	–	–				–	–D				
	910	–	–	–E								
	911	–	–				–		$+^{TN}$E			
	917	–	–		$+^{TN}$E							

– All results negative, + one or more PCR-based methods positive, ^{T} TaqMan PCR positive, ^{R} RT-PCR positive, ^{N} nRT-PCR positive, *E* euthanasia, *D* animal found dead. Blanks indicate that no sampling was performed. Shaded areas indicate that the animal was removed from the experiment

including those taken during the onset of disease and those taken from survivors and in-contact bats, were negative by all PCR-based methods.

Comparative specificity and sensitivity of PCR-based methods

Since TaqMan PCR was our primary method of choice for viral nucleic acid detection and quantitation, all available samples were processed by this method. All swab samples ($n = 313$) were tested by RT-PCR and nRT-PCR, whereas only some tissue samples ($n = 55$) were tested by these additional methods. The results of testing of 368 samples were available to assess the comparative sensitivity and specificity of the PCR-based methods. The RT-PCR was the least sensitive method (25 positive results; 6.8%), whereas the sensitivity of TaqMan PCR (35 positives; 9.5%) held an intermediate position between RT-PCR and nRT-PCR (40 positives; 12.2%). In two cases, TaqMan PCR-positive results were obtained for samples that were negative by all other methods (brown fat from bats #911 and #917, both infected with Irkut virus).

Non-specific amplification occurred during RT-PCR and nRT-PCR. Non-specific PCR products were generally of an improper size, and therefore readily distinguishable from the positive control, with one important exception: eight samples that were negative by TaqMan PCR revealed positive bands by nRT-PCR that were of the desired size (~380 base pairs) and indistinguishable from the positive control. Direct sequencing confirmed that only five of these eight products were specific. Sequence analysis showed the non-specific PCR products to have 83% identity to human chromosomal DNA (GenBank accession no. AP003109). All three samples were derived from oral swabs.

Discussion

The common big brown bat, *E. fuscus*,is a natural host for rabies virus in the United States [18]. This preliminary study has attempted to assess the susceptibility of this host to infection with three recently discovered Eurasian bat lyssaviruses and to assess the suitability of *E. fuscus* as an experimental model for non-rabies lyssavirus infection. Such a study has obvious limitations, many enhanced by the difficulties in performing laboratory infections on a wildlife host. In particular, dose, host susceptibility to a non-adapted virus, and route/site of inoculation should be considered when discussing the generality of these findings.

The principal hosts of Irkut, Aravan, and Khujand viruses are unknown; only one isolate of each virus is currently available [5, 16]. All three viruses were isolated from bats of the *Myotis* and *Murina* genera. Infection dynamics of our experimental host may differ from those of the natural host(s). However, this difference may be limited as all of the bats in question belong to the *Vespertilionidae* family.

RNA virus micro-populations are heterogeneous and genetically labile [10]. This diversity provides a potential for substantial genetic selection following replication in different cellular environments. Our inocula were generated following 2–3 amplification passages in mouse brains, and as such it was important

to assess the possibility of viral adaptation following this procedure. Genetic analysis indicates that the viral consensus sequence was not significantly altered. We compared 5819 nucleotides (including the nucleoprotein, phosphoprotein, matrix protein, glycoprotein genes, and intergenic regions) of Aravan virus after two and seven intracerebral mouse passages. We found only one nucleotide substitution (within the phosphoprotein gene; results not shown). Although this suggests that the genetic structure of inocula were not altered markedly, this does not eliminate the possibility that the micro-structure may have been altered [14]. This limitation is faced in many experimental studies of viral pathogenesis with primary isolates, when original infectious inocula are limited in quantity.

In keeping with the results of earlier studies [4, 15], the susceptibility of bats to lyssavirus infection was found to be moderate: in total, 55–75% of inoculated animals developed rabies. However, the route of infection can alter the dynamics of virus infection of bats [2, 19]. In particular, intramuscular infection appears to reduce the proportion of virus-positive salivary gland compared to the subcutaneous route [2]. Similarly, multiple superficial lesions are favorable for the entry of some plant rhabdoviruses, whereas deeper inoculation is favorable for others [12]. Perhaps a similar situation applies for certain lyssaviruses. Bats are capable of inflicting multiple superficial wounds, and captured *E. fuscus* have numerous small scars suggestive of repeated minor bite wounds [23]. Additionally, bat saliva could contain enzymes (such as hyaluronidase) and other biologically-active components that may alter infection probability.

Infectious virus was detected in brain of all rabid animals, but viral distribution to non-neuronal tissues was limited. In concordance with other studies [4, 15], of all the non-neuronal tissue examined, virus was detected most frequently in salivary gland: in 83.3% of bats that succumbed to Irkut virus and 33.3% of bats that succumbed to Khujand virus. Given the likely mode of transmission of bat lyssaviruses via bite, this is predictable. The absence of infectious virus in the salivary glands of Aravan virus-infected bats may be indicative of poor replicative fitness, more complex virus dynamics, or an experimental artifact.

Virus isolation was successful from one sample of tongue (which may be associated with salivary glands and ducts, or by contamination with saliva), two samples of brown fat, and three samples of lung. Earlier studies have demonstrated low amounts of infectious virus in these organs and tissues (titres $\sim$0.5 $\log_{10}$ $MICLD_{50}$/0.03 ml) [4, 15]. Brown fat has been implicated in the overwintering of RV in *E. fuscus* [24], although this hypothesis is yet to be examined fully. In addition, the presence of EBLV-1 was demonstrated in lungs of naturally infected *E. serotinus* [7]. Since respiratory channels could be considered as conduits of virus excretion in certain unique environments [9], these preliminary observations should be addressed in further studies. The detection of infectious virus (i.e., a positive result by RTCIT) is significantly associated with the levels of viral RNA within the tissue. The small numbers of tissues that were RTCIT positive is most probably due to the lower sensitivity of the infectious assay when compared to TaqMan PCR. There is no reason to suggest that infectious virus was not present

(possibly only at some point prior to death) but at levels below the detection limits of the RTCIT.

The results of TaqMan PCR (and the correlation between TaqMan PCR quantities and the results of RTCIT testing) suggest that spread of Aravan and Khujand viruses to non-neuronal tissues is determined by the level of infection in brain tissue. This relationship does not appear true for bats infected with Irkut virus. Reasons for this observation are currently unclear, although shorter incubation periods for Irkut virus infection may indicate a higher relative replicative fitness and thus more progressive viral spread following infection of the brain. If true, the stage of infection during which the levels of virus in brain directly relate to the extent of extra-neuronal spread may have passed by the time viral replication in brain results in mortality. Of course, the different dynamics of Irkut infection may have been partially determined by the higher dose used in this first set of experiments.

Viral RNA was detected in oral swabs of dying bats 0–2 days before the onset of clinic disease. Taking into account that the duration of clinic disease was limited to 1–2 days, viral RNA could be detected 0–4 days before death. All oral swabs available within 3 days of the first positive swab were negative. Therefore, detectable amounts of viral RNA appeared in saliva no earlier than 5 days before the onset of clinical signs, or within 1 week of death. Viral load in oral swabs was low; virus isolation was only successful from two swabs after two cell culture passages. As observed in other rabies models, shedding of virus may be intermittent, thus reducing the probability of detecting viral RNA in saliva. Altering the parameters of the experiment (dose, route/site of inoculation) should be investigated further before definitive statements can be formulated regarding the kinetics of viral shedding.

Neither infectious virus nor viral RNA could be detected in brain, salivary gland, and oral swabs taken from surviving inoculated and in-contact bats. Although we did not test for serum antibody during these experiments, the presence of virus-specific antibody would indicate either exposure to antigen or replicating virus infection. Therefore, this study is unable to contribute to the hypothesis that bats may be asymptomatic carriers or survive a productive infection with lyssaviruses [11, 21, 26]. Serological testing using micro-neutralization techniques will be applied for future experiments.

Although we could not demonstrate transmission of infection between rabid and in-contact bats, it may be argued that the factors of susceptibility and virus-host adaptation, complemented with the artificial housing and social limitation imposed (in natural colonies bats exist at higher densities and in different micro-environments than those used in the laboratory) may have influenced the lack of contact transmission.

Laboratory testing using PCR-based methods has enabled a comparative assessment of the sensitivity and specificity of those tests to detect viral RNA. In concordance with an earlier study [13], we demonstrated that nRT-PCR is more sensitive than TaqMan PCR. The major advantages of TaqMan PCR lie in the generation of quantitative data with a high specificity that does not require the

confirmation of results by other methods (such as nucleotide sequencing). The three false-positive results in this study were all obtained by nRT-PCR, making up ∼1% of all samples tested by nRT-PCR. The results of RT-PCR and nRT-PCR should therefore be routinely confirmed by sequencing or other available methods.

Clearly, *E. fuscus* is a suitable animal model for studies of the pathobiology of indigenous and exotic lyssaviruses. The species is abundant and widely distributed, accommodates well to captivity, is related to other suggested taxa in Eurasia, and is one of the most common bats submitted for rabies diagnosis in North America. Comparative studies should be initiated with other host species and viral isolates which attempt to compare the influence of viral origin, dose and route of inoculation, host demographics, and environmental conditions on infection dynamics; all to further develop our understanding of the dynamics of pathogen adaptation and host response, as well as the public health significance of emerging infections among the Chiroptera.

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