Novel Methods of Detection and Characterization of RNA Virus Pathogens and their Hosts in the Kyrgyz Republic

B. J. Briggs[1], D. M. Czechowski[1], A. Atoom[1], P. A. Larsen[2], H. N. Meeks[2], J. P. Carrera[2], V. J. Swier[2], R. Hewson[3], B. Atkinson[3], A. T. Junushov[4], O. N. Gavrilova[5], I. Breininger[5], C. J. Phillips[2], and R. J. Baker[2], **J. Hay**[1]

1 Department of Microbiology and Witebsky Center for Microbial Pathogenesis and Immunology, University at Buffalo, Buffalo, New York; 2 Department of Biological Sciences, Texas Tech University, Lubbock TX; 3 Public Health Affairs, Centre for Applied Microbiology and Research, Health Protection Agency, Porton Down, Salisbury, UK; 4 Biotechnology Institute, National Academy of Sciences of the Kyrgyz Republic, Bishkek, Kygyz Republic; 5 Republican Center of Quarantine and Especially Dangerous Infections, Ministry of Healthcare of the Kyrgyz Republic, Bishkek, Kyrgyz Republic

Abstract

RNA viruses belonging to several families are potential hazards for both human and animal populations in Central Asia. In the summer of 2007 we undertook a field trip to the Kyrgyz Republic where we harvested animals and ticks, to initiate establishment of a baseline for viral epizootiology and epidemiology studies. We collected tissues from 185 specimens of rodents and shrews from 4 collecting localities. Prior to this collecting effort, only 8 specimens of mammals from the Kyrgyz Republic were listed in the MaNIS database as archived in accredited museums in the United States. We have generated phylogenetic and phylogeographic results from the rodent genera *Alticola*, *Apodemus*, *Dryomys*, *Microtus*, *Myodes*, and *Rattus* as well as the soricid genus *Crocidura*. Results indicate that the mammalian fauna of the Kyrgyz Republic has complex biogeographic connections with East Asia, South Asia, and East Europe. Our data provide phylogeographic evidence of recent (< 20,000 ybp) colonization events by at least two genera (*Apodemus* and *Crocidura*) into the Kyrgyz Republic. We hypothesize that zoonotic viruses were introduced with colonization and have similarities to pathogens at the geographic origin of the colonizing mammals. In this study we examined the occurrence of viral RNA from hantaviruses, Crimean-Congo hemorrhagic fever virus and tick-borne encephalitis viruses (TBEV) in animals and ticks. Our data suggest that hantaviruses are associated with *Microtus*, *Apodemus*, *Rattus* and *Crocidura* species. We also detected TBEV in *Apodemus pallipes*. The occurrence of hantaviruses in *Apodemus pallipes* has, we believe, never been reported. Using a novel EIA for antibodies based on cloned viral antigens, we have been able to detect antibodies for all three studied viruses in a range of rodents and insectivores. We are currently isolating viral genomes for sequencing. We will correlate viral genomic information with serology and with the phylogeny of rodent and insectivore hosts.

Background

Chinese physicians first described pathologies that resemble hantavirus infection over 1,000 years ago, however isolation of the causative agent was not successful until 1976 when Lee et al. were working in Korea. The association of virus and reservoir rodents seems to be ancient as phylogenies of viruses map closely to groups of rodents (see **Figure 1**). Tick borne encephalitis virus circulates worldwide but causes its most severe pathologies in Russia and Central Europe. The severity of Old-World hantavirus infection varies from the mild nephropathia epidemica (Puumala virus) in Europe, to the severe HFRS (Hantaan virus) in Asia. Much is known about the viruses circulating in South East Asia, the Asian subcontinent, and Europe, however there are no data on hantavirus epidemiology in Central Asia. Due to the unique cultural practices in Central Asia and in view of this region's central role in ancient trade and conquest routes, we set out to characterize the epidemiology and ecology of tick borne encephalitis and hantaviruses in small mammals and humans in Central Asia.

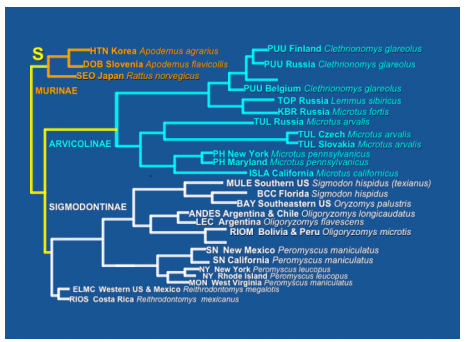

Figure 1. Cladogram of S-segment of various hantaviruses with subfamilies of Cricetidae colored and species of rodent noted after strain of virus. From CDC *"All About Hantaviruses"*.

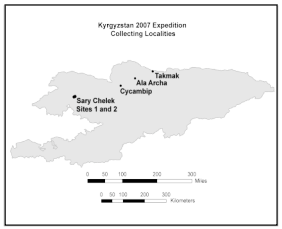

Figure 2. Map of The Kyrgyz Republic showing sites of collection. *From P. A. Larsen*

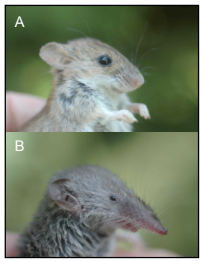

Figure 3. Images of rodent (*Apodemus*) A, and insectivore (*Crocidura*) B. *From R. J. Baker.*

Methods

Serological

A novel EIA based assay was developed using the Nucleocapsid protein (N) of three representative hantaviruses (Puumala, Tula, and Hantaan). Each recombinant N was expressed either in an E. coli or baculovirus expression system and purified by affinity chromatography. A tick borne encephalitis E protein construct in a pMal expression system was used for TBE serological assays, courtesy of Holbrook et. al.. The recombinant antigen was bound to 96 well plates. Whole blood from rodent samples was applied to Nobuto filter paper, followed by elution in PBS. These samples were then used for EIA. Detection of reactive IgG was performed using a rabbit anti-mouse IgG-HRP and Protein-G-HRP conjugates from Sigma. Samples were normalized by Bradford protein assay.

Nucleic Acid Detection

Tissues (Spleen, Liver, Lung, Submandibular Salivary Gland) were isolated from each rodent in the field. These selected tissues were applied to FTA (Whatman) cards as per the manufacture's recommendations. 2mm punches were then taken from the FTA cards and RNA was eluted using the Whatman protocol for RNA isolation from FTA. RNA eluted from FTA was then used for detection of hantaviral nucleic acid in a LightCycler 2.0 (Roche) using published protocols.

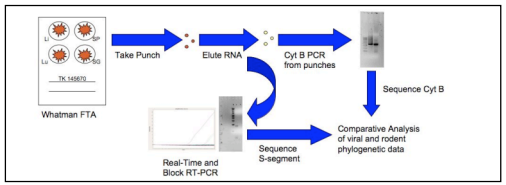

Figure 4. Rationale and procedure for RNA and DNA isolation from FTA

Results

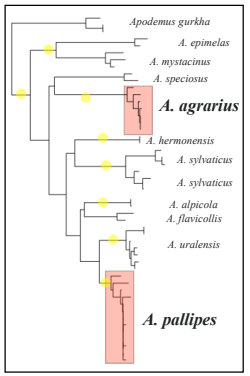

Figure 5. Phylogram of *Apodemus sp.* based on CytB sequence. Shaded clades represent those *Apodemus sp.* collected on our expedition. *A. pallipes* showed a <1% divergence within Kyrgyzstan and ~2% divergence from samples collected in Nepal. *A. agrarius* has <2% divergence from South Korean samples and <1% from Eastern European samples. The unexpected low amount of genetic distance both within our data set and between our data set and those rodents sampled elsewhere in Asia suggest a recent colonization of Central Asia, likely within the last 20,000 years. *From P. A. Larsen.*

Field Identification	Number	Hanta	TBE
Alticola argentatus	2	0, 0	1, 0
Apodemus pallipes	79	2, 7	3, 5
Apodemus agrarius	11	0, 1	0, 0
Crocidura suaveolens	10	2, 1	0, 0
Crocidura leucodon	1	0, 0	0, 0
Dryomys nitedula	11	0, 0	0, 0
Microtus arvalis	39	2, 0	3, 0
Mus musculus	3	0, 3	0, 1
Myodes sp.	1	0, 0	0, 0
Rattus turkestanicus	26	3, 1	0, 1
Rattus norvegicus	1	0, 0	0, 0
Total	184	9, 13	7, 7

Table 1. Overview of total samples collected on expedition with positive real-time (Red) and serology (Green) shown for each tested virus.

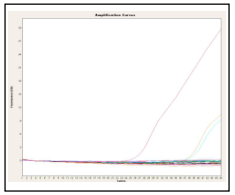

Figure 6. Representative output of real-time RT-PCR analysis. Positive curves are TK 146528 and TK 146534. *From B. Atkinson.*

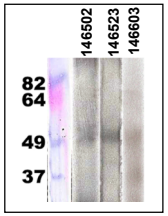

Figure 7. Western blot of three samples found positive by EIA. Protein-G-HRP used for detection.

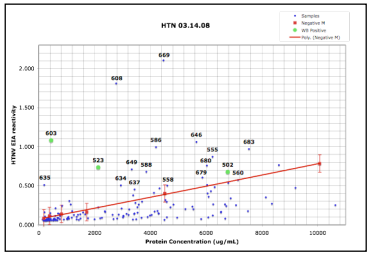

Figure 8. Representative graphic of Hantaan virus EIA/Bradford analysis. Those data lying above the 95% CI of negative controls were counted as positive. Solid green circles are positive by western blot.

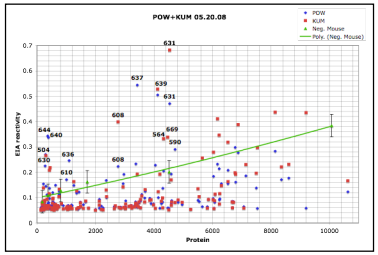

Figure 9. Representative graphic of TBEV EIA/Bradford analysis. Those data lying above the 95% CI of negative controls were counted as positive.

Discussion

- These methods have allowed us to detect novel evidence of hantaviruses and TBEV circulating in Central Asia, specifically the Kyrgyz Republic; this is the first such report
- Some samples are positive for serology but are negative for RT-PCR-either these samples are no longer shedding virus or our current RT-PCR assays are not able to pick up these viruses
- Some samples are positive for viral RNA yet are not positive by our serological assays-it is possible that these individuals are early in infection and have not started producing IgG
- We have strong evidence of hantaviruses and TBEV circulating in *Apodemus pallipes*-this is entirely novel as, to date, there has been no evidence that *A. pallipes* carry any viruses
- There are preliminary data that suggest shrews are infected with puumala and/or tula like hantaviruses this has not been reported
- TBEV appears to cluster geographically into "hot spots", IgM detection will identify localities where TBE is likely to be in circulation

Future Directions

- Optimize block RT-PCR assay so that we will be able to amplify portions of the S-segment for sequence analysis
- Return to the Kyrgyz Republic to sample from localities and populations that have been identified in these preliminary studies
- Due to limitations of Nobuto strip technology, collect serum instead of using elution of whole blood from Nobuto strips
- Integrate detection of IgM into our current IgG EIA assay
- Collect more human serological specimens for analysis with our EIA assay