Evidence from nature: interspecies spread of heron hepatitis B viruses

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Received 26 November 2004
Accepted 18 January 2005

Heron hepatitis B viruses (HHBVs) in three subspecies of free-living great blue herons (Ardea herodias) from Florida, USA, were identified and characterized. Eight of 13 samples were positive in all assays used, whereas sera from egrets, which are also members of the family Ardeidae, were negative in the same assays. Comparative phylogenetic analysis of viral DNA sequences from the preS/S region of previously reported and novel HHBV strains isolated from captive grey herons (Germany) and free-ranging great blue herons (USA), respectively, revealed a strong conservation (95% sequence similarity) with two separate clusters, implying a common ancestor of all strains. Our data demonstrate for the first time that different subspecies of herons are infected by HHBV and that these infections exist in non-captive birds. Phylogenetic analysis and the fact that the different heron species are geographically isolated populations suggest that lateral transmission, virus adaptation and environmental factors all play a role in HHBV spreading and evolution.

INFORMATION

Hepatitis B viruses (HBVs) are small, enveloped DNA viruses belonging to the family Hepadnaviridae, which propagate their genome by reverse transcription of an RNA intermediate. As pararetroviruses, they are classified together with members of the families Retroviridae and Caulimoviridae (Mayo & Pringle, 1998). The family Hepadnaviridae comprises the genera Orthohepadnavirus (infecting mammals) and Avihepadnavirus (infecting birds).

Hepadnaviruses are generally known for their rather narrow host range. For instance, human HBV infects only human, chimpanzee (Pan troglodytes), chacma baboon (Papio ursinus orientalis) and, to some extent, tree shrew (Tupaia belangeri) hepatocytes (Yan et al., 1996; Guidotti et al., 1999; Kedda et al., 2000; Takahashi et al., 2000). Woodchuck hepatitis B virus (WHV), another example of an orthohepadnavirus, has only been detected in Eastern woodchucks (Marmota monax) and does not infect the related alpine marmosets (Marmota marmota) (Tyler et al., 1981; Chomel et al., 1984). Ground squirrel hepatitis B virus (GSHV) infects only some species of squirrel (Spermophilus beecheyi and Spermophilus richardsonii) and the phylogenetically closely related chipmunks (Eutamias spp.) (Trueba et al., 1985). A closely related virus (arctic squirrel hepatitis virus; ASHV) was identified in arctic ground squirrels (Testut et al., 1996), but its host range has not been studied so far.

Avihepadnaviruses have been detected in various duck species [Anas spp.; duck hepatitis B virus (DHBV); reviewed by Schödel et al., 1991; Triyatni et al., 2001], snow geese [Anser caerulescens; snow goose hepatitis B virus (SGHBV); Chang et al., 1999], a Ross’ goose [Anser rossii; Ross’ goose hepatitis B virus (RGHBV); GenBank accession no. M95589], white storks [Ciconia ciconia; stork hepatitis B virus (STHBV); Pult et al., 2001], demoiselle and grey crowned cranes [Anthropoides virgo and Balaeniceps regulusum, respectively; crane hepatitis B virus (CHBV); Prassolov et al., 2003], and grey herons [Ardea cinerea; heron hepatitis B virus (HHBV); Sprengel et al., 1988; Netter et al., 1997]. Like their mammalian counterparts, avihepadnaviruses have a rather narrow host range. For instance, DHBV

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infects only certain duck species, but does not infect Muscovy ducks or chickens (Schödel et al., 1991; Sprengel et al., 1991; Pugh & Simmons, 1994). Little is known about the host range of HHBV or STHBV. Despite its substantial sequence similarity to DHBV, HHBV does not infect ducks and only infects primary duck hepatocytes very inefficiently (Sprengel et al., 1988). Recently, it has been reported that cranies are naturally infected with a hepadnavirus, designated CHBV (Prassolov et al., 2003). Cranies are phylogenetically very distant from ducks and are more closely related to herons and storks. Interestingly, however, CHBV infects primary duck hepatocytes with an efficiency similar to that of DHBV. Collectively, these and related data suggest that the host range of hepadnaviruses cannot be simply predicted based on the evolutionary relatedness of their respective hosts.

Naturally occurring DHBV infections have been reported in Pekin ducks (Anas domesticus) and related species from China, USA, Canada, Europe, India and South Africa (Schödel et al., 1991; Munshi et al., 1994; Triyatni et al., 2001; Mangisa et al., 2004). Phylogenetic analysis of the various isolates demonstrated a rather high variability among DHBV strains, whereas genomes from other avian hepadnaviruses identified so far, such as STHBV and CHBV, appear less variable (Sprengel et al., 1988; Netter et al., 1997; Triyatni et al., 2001; Pult et al., 2001; Prassolov et al., 2003).

Species specificity of hepadnaviruses seems to be, at least to some extent, determined at the level of virus entry, involving the preS part of the large envelope protein L. Substitution of the N-terminal region (aa 22–90) of the HHBV-specific preS domain with the corresponding sequence from DHBV renders HHBV infectious for ducks and is therefore sufficient to overcome the species barrier of HHBV in primary duck hepatocytes (Ishikawa & Ganem, 1995). Accordingly, comparative genomic and subgenomic sequence alignment from different avian hepadnaviruses facilitates prediction of the specific properties of each virus and helps to gain insight into the mechanisms controlling species specificity and host adaptation.

DNA sequences from full-length genomes of the few HHBV isolates reported diverge from those of DHBV by >20%. The preS region of the HHBV L protein is hypervariable. It has the lowest sequence similarity to all hepadnaviral proteins, diverging by about 50% from the DHBV protein (Sprengel et al., 1988, 1991; Netter et al., 1997). However, the biological significance of this divergence is not yet clear. This is partly because only a few cloned HHBV full-length genome sequences are available, which have all been derived from captive herons in German zoos. Based on the available data, it is not clear whether HHBV infection occurs at all in free-living herons, as cross-species virus transmission in zoos cannot be excluded as a possible source of these reported infections.

Here, it is shown that HHBV infection occurs not only in captive grey herons, but also with high prevalence in free-living birds. HHBV has been detected in another heron species, the grey blue heron (Ardea herodias), as well as in two of its subspecies (great white heron and Würdemann’s heron). Thus, the data shown here demonstrate for the first time the occurrence of this virus in free-living birds and show that HHBV is an endogenous virus of several heron species.

### METHODS

#### Animals and sera.
Samples tested included sera from great blue herons (n = 7), great white herons (n = 5) and Würdemann’s heron (n = 1). Additionally, five serum samples from great egret (Egretta alba), which are members of the same family (Ardeidae), and two serum samples from great cormorants (Phalacrocorax carbo), which are members of a different family (Phalacrocoracidae) and order (Pelecaniformes), were analysed. All sera were collected as part of the standard procedure for evaluating free-living injured birds, which were brought to the Florida Keys Wild Bird Centre in Tavernier, FL, USA, for treatment and rehabilitation.

#### PCR amplification and sequencing of viral DNA from serum.
Sera were screened for viral genomes without prior DNA extraction. One microlitre of native serum was diluted in 199 μl lysis buffer [50 mM KCl, 0-45 % (v/v) Tween; 0-5% (v/v) NP-40; 10 mM Tris/HCl (pH 8-3)] and subjected to protease K digestion at 56°C for 2 h. Thereafter, protease K was inactivated by heating at 95°C for 10 min. For amplification of the complete HHBV genome, 5 μl digested sample was subjected to PCR using HHBV-4-specific full-length primers, which anneal to the so-called nick region of the viral DNA. Primer F1 aligns to nt 2539–2561 (5′-GAAGATCT-GCTCTCATTACACCCCTCCTCATTGGCGGC-3′) and the reverse primer R1 to nt 2519–2541 (5′-GAAGATCTGCTCTTCAATCTTAGAGACACATAGCT-3′). PCR was carried out by using the Expand High Fidelity PCR system (Roche) as described previously (Netter et al., 1997; Prassolov et al., 2003). Amplified PCR products were analysed on an ethidium bromide-stained 1% agarose gel, purified by using a QIAquick Gel Extraction kit (Qiagen) according to the manufacturer’s instructions and sequenced directly.

The subgenomic sequence containing the preS/S ORF was amplified by using purified full-length genome as template and primers F2 annealing to nt 772–789 (5′-GGCGGCGCGGCGCATGTTGGC-GCAAAC-3′) and R2 annealing to nt 1801–1821 (5′-GGCGGCGGCGCATTTTGTCAAAGTTGATC-3′) of HHBV 4, containing a heterologous restriction site for NolI. PCR products were digested with NolI and subcloned into the vector pcDNA3 (Invitrogen). Sequencing of cloned pcDNA3-HHBV-preS was carried out by using an ABI BigDye Terminator Cycle Sequencing kit. Sequencing PCR was performed by using primers T7 (5′-TAATACGACACTCACTATAGG-3′) and SP6 (5′-ATTTAGGTGACACTATAGAA-3′). Sequence analysis was performed using the 3100 Genetic Analyser (ABI) and MACVECTOR software.

To calculate sequence divergence values among the different viruses, the software MEGA 2.1 (Kumar et al., 2001) was used. Phylogenetic relationships and genetic distances of the virus genomes were estimated by using neighbour-joining (NJ) algorithms implemented by MEGA. Bootstrap analyses were based on 1000 replications. The significance of the branch lengths in the NJ tree was examined by a standard error test using the confidence probability. The phylogenetic network was constructed by using the median-joining (MJ) algorithm of NETWORK 4.0 (Bandelt et al., 1999). MJ networks include all most-parasimonious trees supported by the data and are particularly appropriate for the detailed resolution encountered in closely related intra- or interspecific datasets.
**SDS-PAGE and immunoblotting.** Immunoblot analysis was performed for detection of viral L antigen in serum samples by using antisera, as described previously (Pult et al., 2001; Prassolov et al., 2003). An aliquot of each serum sample (1 μl) was diluted in 49 μl PBS (pH 7). Samples were denatured in 50 μl 2 × Laemmli buffer and boiled at 99 °C for 5 min. Each sample (20 μl) was separated by 15 % denaturing SDS-PAGE and the proteins were transferred to nitrocellulose membranes. The membranes were then blocked for 1 h at room temperature in 5 % dry milk dissolved in TBST (Tris-buffered saline, 50 mM Tris/HCl, 150 mM NaCl, 0–1 % Tween 20) and incubated overnight at 4 °C with an HHBV preS-specific rabbit antiserum at a 1:2000 dilution. After several washings with TBST, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Dianova) at a dilution of 1:20000. Proteins were visualized by chemiluminescence (SuperSignal West Dura; Pierce).

**Electron microscopic analysis of virus particles.** Electron microscopy was performed to obtain ultrastructural evidence for hepadnavirus particles in the respective bird sera. The great blue heron serum analysed was positive, as assayed by immunoblotting and PCR, whereas the control serum was negative in both assays. Heron sera were incubated with micro-carriers pre-coated with HHBV preS antiserum. After several washings, the carriers were pelleted by low-speed centrifugation, washed, resuspended in PBS and subsequently transferred into capillary tubes, as described previously (Hohenberg et al., 1994; Pult et al., 2001). For electron microscopic analysis, samples were fixed with 2-5 % glutaraldehyde in PBS for 1 h at room temperature, washed and post-fixed for 30 min with 1 % OsO4 in PBS. For ultrathin sectioning, the samples were gradually dehydrated with ethanol and embedded in ERL resin. Ultrathin sections were counterstained with 2 % uranyl acetate and lead citrate. All electron micrographs were obtained with a Philips CM120 transmission electron microscope at 60 kV.

**RESULTS**

**High prevalence of HHBV-related genomes and envelope antigens in sera of herons and related species from the USA**

In total, 13 serum samples were collected from seven great blue herons, five great white herons and one Würdemann’s heron (FL, USA) and screened for the presence of HHBV. By using both PCR (Fig. 1) and immunoblotting (Fig. 2), HHBV infection was shown to occur in three of the seven great blue herons, four of the five great white herons and the Würdemann’s heron (Table 1). In contrast, no evidence for HHBV infection was found in the five egret or two great cormorant samples that were also tested in the same assays (Figs 1 and 2; data not shown). Full-length PCR amplification products obtained from heron serum samples were indistinguishable in size (3-0 kb) from that of a known HHBV isolate (Fig. 1, lanes headed P). By using immunoblotting, L envelope proteins identical in electrophoretic mobility to those from an HHBV-viraemic grey heron serum were detected (Fig. 2). Notably, the HHBV-positive control serum (from a grey heron) showed a stronger signal in the immunoblot compared with those seen with any of the sera tested. Data obtained by two independent assays (PCR and immunoblot) strongly indicated infection of three different heron species other than grey heron with HHBV-related hepadnaviruses.

**Electron microscopic detection of HHBV particles after immunocapture from viraemic sera**

Direct proof for the presence of HHBV-related virus and subviral particles in heron sera was obtained by electron microscopy. Viral particles were adsorbed onto beads by using HHBV envelope-specific antisera and analysed thereafter by electron microscopy. Subviral particles with a
diameter of 40–60 nm were mainly observed (Fig. 3). They were indistinguishable from other avihepadnavirus particles described previously (Pult et al., 2001; Prassolov et al., 2003) and were present only in a viraemic serum previously shown to be positive by PCR and L immunoblot assays (Figs 1 and 2).

Comparative analysis of the L protein sequence of the new HHBV isolates

Cycle sequencing of the HHBV L protein-encoding region in the viral genome of one representative from each subspecies of heron [GenBank accession nos AY519979 (HHBV 45), AY519980 (HHBV 43) and AY519981 (HHBV 5)] revealed a high degree of similarity (approx. 95 %) with the four HHBV isolates reported previously in grey herons (Fig. 4; Netter et al., 1997). However, several nucleotides and amino acids are unique in the new USA isolates. For example, in the preS region of the L protein, the amino acid sequence differed by five residues (Q23H, Y43F, I139L, Q162T/P and T163N), whereas the S region contained only two amino acid exchanges (R211K and I329L). Residues 22–90 of the preS region, previously identified as the host range-determining domain of avian hepadnaviruses (Ishikawa & Ganem, 1995), contained only a single amino acid exchange (Q23H). This raises the possibility that all HHBV isolates may have a compatible host range.

The predominantly conservative nature of the preS sequence at aa 34–119, which was reported to be essential for binding to CPD (gp180), the putative cellular receptor, is consistent with its proposed role in virus entry (Urban et al., 1998; Urban & Grip, 2002).

Although the sequence comparison revealed that the new sequences are heron-specific, phylogenetic analysis of all avihepadnaviral L protein sequences demonstrated the existence of a second cluster of HHBV, clearly distinct from the HHBV sequences described so far (Fig. 5). Network correlations showed substructuring within the HHBV isolates from Germany. HHBV A is derived directly from HHBV B and all three HHBV isolates (HHBV A, HHBV B and HHBV 4) are derived from the same ancestor. HHBV C and HHBV D are also related very closely. Furthermore, this analysis revealed that HHBV isolates from Florida have a basal position to the German isolates and that HHBV isolates 5 and 45 were derived from HHBV isolate 43 (Fig. 6).

DISCUSSION

In this study, a high prevalence of HHBV was found in free-living heron species/subspecies other than those described previously and in a new geographical area (FL, USA). The detection of HHBV-related nucleic acids, viral envelope proteins and virus particles provided unequivocal evidence for the natural infection of these animals with HHBV. Furthermore, the number of positive animals (7 out of 13) suggests that up to 50 % of free-living herons in North America may be naturally infected with HHBV, which further suggests a high prevalence of HHBV infection in these birds. The fact that some samples showed only weak signals in immunoblots is most probably due to a lower viraemia in the herons tested. The alternative explanation of a lower immunoreactivity of the HHBV preS antiserum with the corresponding proteins seems most unlikely, due to the strong conservation of the preS/S sequence of the HHBV isolates. A comparison between new and previously reported HHBV isolates and the conservative nature of most amino acid exchanges in the L protein strongly suggest a similar structure of HHBV envelope proteins and infection mechanism in various heron species. HHBV is clearly distinct from avihepadnaviruses isolated from snow geese (SGHBV), Ross’ goose (RGHBV), white

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**Table 1. Animals investigated in this study**

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<th>Order</th>
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<th>Genus</th>
<th>Species</th>
<th>Common name</th>
<th>No. animals</th>
<th>No. positive for HHBV</th>
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<td>Ardea</td>
<td>Ardea herodias</td>
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<td>Ardea</td>
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<td>Great white heron</td>
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<td>4</td>
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<td>Ardea</td>
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<td>Würdemann’s heron</td>
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<td>1</td>
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<td>Great white egret</td>
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<td>0</td>
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<tr>
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<td>Phalacrocorax carbo</td>
<td>Great cormorant</td>
<td>2</td>
<td>0</td>
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</tbody>
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**Fig. 3.** Ultrastructural evidence for hepadnaviral particles in the serum of a great blue heron. Immunocaptured virus particles with a diameter of 40–60 nm are shown at a lower (a) and a higher (b) magnification. Bars, 100 nm.
storks (STHBV), cranes (CHBV) and ducks (DHBV). The host range-determining sequence motif at the N-terminal end of the L protein of all HHBV isolates is very similar. As the WTP motif in the DHBV preS region, which was shown previously to be critical for hepatocyte infection (Sunyach et al., 1999), is changed to WTE in all of the HHBV isolates (Netter et al., 1997; this study), it seems likely that HHBV infects only heron and possibly closely related species. In our study, however, naturally occurring HHBV or related viruses were not detected in egrets of the same geographical area, suggesting species barriers to infection. Alternatively, this may be due to the low number of samples available and further analysis may be required.

DHBV isolates cluster in at least two groups (Asian isolates, including Australia, versus isolates from the USA and Europe). The majority of DHBV strains from Asia were isolated from closely related duck species and are less conserved than the few USA/European strains isolated from distinct species. Genomes from HHBV, SGHBV, STHBV and CHBV were conserved and not divided into subclusters (Triyatni et al., 2001; Prassolov et al., 2003). Accordingly,
the higher diversity of DHBV strains was considered to be unusual in avihepadnaviral evolution, but data regarding other avian HBV-like viruses were limited and restricted the extent of comparative analysis. All isolates of HHBV analysed thus far were from captive grey herons from northern Germany (Sprengel et al., 1988; Netter et al., 1997). Likewise, STHBV was isolated and sequenced from captive white storks from Germany (Pult et al., 2001) and the isolates from snow geese were all collected from one zoo in Germany (Chang et al., 1999). Interestingly, the CHBV isolates were obtained from two different species (Anthropoides virgo vs Balearica regulorum) and subfamilies (Gruinae vs Balearicinae), but again were all derived from German zoos. Therefore, lateral transmission of CHBV in captivity cannot be excluded so far.

As hepadnaviruses replicate via an RNA intermediate and error-prone reverse transcription, the low variability in most avihepadnaviral genomes was unexpected. The highly conserved nature of HHBV, even among viruses isolated from different heron species (Fig. 4), suggests that host specificity and/or geographical barriers have a strong impact on the evolution of new viruses. Phylogenetic analysis of the HHBV L gene demonstrates that the HHBV strains from grey (Germany, Europe) and blue (Florida, USA) herons form separate clusters, suggesting that regional aspects may also be important for understanding the evolution of HHBV (Fig. 5). Thus, our study adds important information and a new perspective on avihepadnaviral divergence, spreading and evolution. In nature, however, DHBV spread occurs by vertical transmission; horizontal transmission of avian hepadnaviruses has not been demonstrated conclusively so far.

Our data demonstrate that the previously isolated HHBV strains from captive grey herons (from German zoos) (Netter et al., 1997) are related closely to the HHBV strain infecting the free-ranging birds from North America. Phylogenetic analysis of all HHBV isolates (Fig. 6a) shows clearly that the North American and European isolates of HHBV are related sister clades that share a common ancestor. The close sequence identity (the DNA sequences coding for the L protein of HHBV 4 and the new isolate HHBV 43 are approx. 95% identical), the ancestral position of the US isolates (Fig. 6a) and the high rate of infection found in herons from Florida suggest that the captive European grey herons were infected by natural, horizontal or iatrogenic transmission with an ancestral virus of these US heron species that has evolved since by continued transmissions in the zoo setting. Taking into account the

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**Fig. 5.** HHBV isolates from wild herons form a separate cluster. The phylogenetic relationship of avian hepadnaviruses was analysed based on L protein sequences by NJ analysis (p-distance). GenBank accession numbers are indicated in parentheses. Bootstrap values are provided for the major nodes of heron sequences (percentage support; 1000 replicates). It is notable that the new HHBV isolates from USA form a separate cluster.
short history of zoological gardens and the animal trade, it is assumed that formation of the two clusters is less than 150 years old. At present, it cannot be formally excluded that HHBV 4 or closely related strains are prevalent in European wild grey herons and that these strains may resemble intermediate strains in the evolution of HHBV. To clarify this possibility, analysis of viruses isolated from wild herons from Europe must be performed to see whether these animals are an alternative source of infection for zoo animals.

Although the grey and great blue heron evolved a long time ago and today exist allopatrically as geographically isolated populations, some ornithologists consider them as ‘con-specific’ or allied together as a ‘superspecies’. Such a close relationship would be compatible with the high sequence identity of the viruses from the two sister clades. From this point of view, an alternative explanation arises in that the HHBV genome sequences may be constrained and do not change as readily as DHBV in domesticated ducks. The two lineages of HHBV would then still have a common ancestor, but the ‘node’ would have to date back more than 100,000 years before the last ice age, which seems rather unlikely according to our current knowledge of the virus life cycle and evolution dynamics.

In conclusion, the existence of HHBV in a free-living heron species has been shown, thereby expanding the current knowledge of naturally occurring avipadenaviruses infections. The present data argue against a monophyletic origin of avipadenaviruses, as well as strict co-evolution of the viruses and their hosts. To determine the importance of the host–species interaction, geographical restriction and adaptation in avipadenaviral evolution, further studies on closely related heron species or other *Ardea* species in different geographical regions need to be performed.

Fig. 6. Phylogenetic analysis of HHBV isolates. Detailed analysis using L protein sequences of HHBV isolates and STHBV 7 as outgroup. Phylogenetic (a) and network (b) analyses demonstrate that the HHBV isolates described here are ancestral to the HHBV isolates described earlier. Additionally, HHBV 5 and HHBV 45 developed directly from HHBV 43. The small nodes (light grey dots) represent not sampled, possibly extant genotypes or extinct ancestral sequences.

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Interspecies spread of heron hepatitis B viruses
ACKNOWLEDGEMENTS

The authors thank O. Krone for helpful discussions and C. Pitra for support with the phylogenetic analysis. We thank S. Kinkley for critical reading of the manuscript. The Heinrich-Pette-Institute is supported by the Freie und Hansestadt Hamburg and the Bundesministerium für Gesundheit und Soziale Sicherung. The Institute for Zoo and Wildlife Research is supported by the city of Berlin and the Bundesministerium für Bildung und Forschung (BMBF). This work was supported by grants from the DFG and the BMBF (Hepnet and NGFN). L. L. is supported by a DAAD fellowship.

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