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What can we learn about lyssavirus genomes using 454 sequencing?

Was können wir über Lyssavirus-Genome mittels 454-Sequenzierung lernen?

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Summary

The main task of the individual project number four "Whole genome sequencing, virus-host adaptation, and molecular epidemiological analyses of lyssaviruses" within the network "Lyssaviruses – a potential re-emerging public health threat" is to provide high quality complete genome sequences from lyssaviruses. These sequences are analysed in-depth with regard to the diversity of the viral populations as to both quasi-species and so-called defective interfering RNAs. Moreover, the sequence data will facilitate further epidemiological analyses, will provide insight into the evolution of lyssaviruses and will be the basis for the design of novel nucleic acid based diagnostics. The first results presented here indicate that not only high quality full-length lyssavirus genome sequences can be generated, but indeed efficient analysis of the viral population gets feasible.

Keywords: next generation sequencing, deep sequencing, rabies, defective interfering particle

Zusammenfassung

Hauptziel des Teilprojektes 4 mit dem Titel „Whole genome sequencing, virus-host adaptation, and molecular epidemiological analyses of lyssaviruses" innerhalb des Forschungsverbundes „Lyssaviruses – a potential re-emerging public health threat" ist es, vollständige Lyssavirus-Genomsequenzen mit hoher Qualität zu erstellen. Diese Sequenzen werden im Hinblick auf die Zusammensetzung der Virenpopulation hinsichtlich Quasispezies und des Vorkommens sogenannter „defective intefering RNAs" detailliert untersucht. Darüber hinaus werden die gewonnen Sequenzinformationen für epidemiologische Analysen herangezogen und Aussagen über die Evolution der Lyssaviren erlauben. Weiterhin dienen die Genomsequenzen als Basis für die Entwicklung neuer Nukleinsäure-basierter Diagnostikmethoden. Die ersten hier dargestellten Ergebnisse des Projektes zeigen bereits, dass nicht nur die Gewinnung qualitativ hochwertiger Genomsequenzen, sondern auch die Analyse der Diversität der Viruspopulation möglich ist.

Schlüsselwörter: next generation sequencing, deep sequencing, Tollwut, defective interfering particle

Introduction

The most prominent species within the genus *Lyssavirus* are those which cause rabies. Only with the advent of modern molecular techniques, i. e. nucleotide sequencing, the delineation of lyssaviruses into genotypes (species) became possible. Partial sequence analysis has also fundamentally increased the understanding of the epidemiology of lyssaviruses in time (e. g. occurrence) and space (e. g. disease spread) (Brookes et al., 2004). With the availability of full-length genome sequences not only the robustness of phylogenetic analyses is increased but these sequences are also the prerequisite for the design of reliable nucleic acid based diagnostics. Besides speeding up the generation of such full-length genome sequences, recent advances in DNA sequencing technologies even offer more: The immense sequence depth achieved and the possibility to randomly start sequencing of nucleic acids offer the possibility to detect structural variations. Both quasi-species analysis and analysis of the structure of defective interfering genomes are becoming feasible. Here we present first results of our efforts to establish next generation sequencing both for routine and in-depth analysis of lyssaviruses.

454-Sequencing

Sequencing within this project is mainly done with a Roche/454 Genome Sequencer FLX platform. This Next Generation Sequencing (NGS) technology is based on the pyrosequencing approach initially developed by Ronaghi and colleagues (1996). For the Genome Sequencer FLX, this technique was optimized to yield longer sequence reads (single sequence strings of up to 500 base pairs) and to run as a highly parallelized process (Margulies et al., 2005). The key features of the Genome Sequencer FLX technology are the sample preparation without cloning (unlike the Sanger based shotgun approach; Sanger et al., 1977), the clonal amplification of a complete DNA library in an emulsion PCR (emPCR) and the massively parallel sequencing of the bead bound shotgun library in the wells of a Pico Titer Plate (PTP) yielding one sequencing read per bead. With a single instrument run approximately one million high quality sequencing reads with up to 500 Mb of raw sequence can be generated. Every single sequence read reflects one original independent input DNA or cDNA, i. e. RNA, molecule. Thereby, in one experiment several independent sequencing repetitions are achieved. These different DNA molecules are generated by random fragmentation of the DNA (or RNA in the case of cDNA libraries) during preparation of the sequencing libraries. Special adapters which serve as priming sites for PCR amplification of the library are ligated to the library fragments. These adapters eliminate the need for cloning into plasmid vectors or sequence knowledge prior to sequencing. Via one of these adapters the library fragments are bound to beads during clonal amplification in an emPCR. In the emPCR, the water droplets of the water in oil emulsion are micro-reactors. These micro-reactors contain the PCR reagents, one library fragment, and a library bead that finally carries the library fragment amplicon. Thereby, in the emPCR millions of different library fragments can be clonally amplified in a single PCR. After emPCR, the library beads are purified from the emulsion and are subsequently enriched. After this enrichment, the beads are prepared for sequencing and loaded into a PTP. This PTP has millions of wells which only hold one bead each. The resulting separation of the single library carrying beads and their fixation in the wells of the PTP during the sequencing allows for parallel sequencing of the bead bound amplicons as single sequence reads.

Once the sequencing is finished, the millions of reads have to be combined into larger contiguous sequences, so called contigs. A contig is a set of overlapping sequencing reads that can be aligned to yield a longer consensus sequence made up of the different reads. Within this alignment which is the basis for the generation of the consensus sequence, the individual reads (which all reflect individual pieces of input DNA/cDNA) retain their individual divergences from the consensus. If divergences are concordantly found in several individual reads, one may assume that these differences between groups of reads and the consensus sequence are truly part of the virus population.

Targeting the shotgun sequencing process

In a typical nucleic acid preparation extracted from a cell culture or tissue sample, the ratio of viral nucleic acids is usually below 1% and rarely reaches 5%. In the shotgun sequencing approach described above, for every nucleic acid molecule in the input material the probability to become sequenced is equal. Hence, preparation of a simple shotgun sequencing DNA/cDNA library, although a straightforward approach, will yield extremely huge amounts of host instead of viral sequences. Therefore, for full-length virus genome sequencing it is necessary to target the sequencing process away from host nucleic acids to viral nucleic acids in order to maximize the output of viral sequences. Until now, diverse techniques have been used for this purpose (Höper et al., 2009; Huang et al., 2009; Potgieter et al., 2009; Monger et al., 2010; Höper et al., 2011). The mentioned techniques include separation of virions from host cell components by centrifugation, enrichment of viral nucleic acids by probing either host or viral nucleic acids, or targeting the viral genomes by PCR. As the different techniques require different amounts and qualities of sample input, not every method is suitable for every sample. Therefore, in order to maximally benefit from the sequencing data the unbiased sequencing with the Genome Sequencer FLX generates, proper sample preparation prior preparation of the sequencing DNA libraries is of utmost importance. To this end, different strategies of sample preparation have been established and will be evaluated in the course of this project. Those that turn out to be suitable for lyssavirus sequencing will further be optimized in order to have the proper targeting approach for each sample type at hand. Once the targeting of the sequencing away from host to the viral nucleic acids works, we will be able to fully benefit from the high amount of raw data to yield maximum output of lyssavirus sequences and information about genome structure.

Lyssavirus genomes

The genus *Lyssavirus* belongs to the family *Rhabdoviridae* within the order *Mononegavirales*. These viruses have a negative stranded monopartite RNA genome. Rabies virus is the prototype of the lyssaviruses. Its genome has an approximate length of 12 000 nucleotides. It comprises five open reading frames (ORFs; depicted in Figure 1, panel A) coding for the nucleoprotein (N), the phospo-

protein (P), the matrixprotein (M), the glycoprotein (G), and the viral RNA-dependent RNA-polymerase (L). To render gene expression from the genome possible, at the 3′ end before the start of the N gene a leader sequence is necessary. In addition, between the open reading frames signals are present to govern transcription stop, polyadenylation, and transcription restart. Moreover, at the 3′ and 5′ ends of the genome, respectively, the genome and anti-genome promoters are located. These serve as initiation sites for genome replication.

Intensive research is conducted in order to relate the different pathogenicity of various virus strains to the differing protein variants they comprise (for a recent overview of research in this field please refer to Jackson, 2011). Full-length genome sequences are the basis for comparative sequence analysis and subsequent shuffling of genes from different viruses by reverse genetics approaches. Via reverse genetics systems, the different variants of the proteins can be combined arbitrarily to try to figure out the impact of different protein variants on pathogenicity. Beyond the possibility to compare strains of different pathogenicity with regard to their respective proteome, in-depth full-length genome sequencing opens up the possibility to analyse the diversity of a virus population.

Diversity of viral genomes

The virus population with its more or less diverging genome sequences is referred to as the so-called quasi-species. According to Eigen and Schuster (1977) "a quasi-species is defined as a given distribution of macromolecular species with closely interrelated sequences, dominated by one or several (degenerate) master copies". The minor portion of mutant sequences may have single or multiple deviations from the consensus sequence in different combinations. The diversity of the quasi-species confers high adaptability to the virus (reviewed in Domingo and Holland, 1997). The impact of the quasi-species on viral pathogenicity was shown, for instance, for poliovirus for which a diverse quasi-species is indispensable for full pathogenicity (Vignuzzi et al., 2006). In the case of lyssaviruses, there are only very few reports on quasi-species analyses (Morimoto et al., 1998; Kissi et al., 1999). One reason for the lack of knowledge about lyssavirus quasi-species may be the technical problem that is posed by traditional Sanger sequencing (Sanger et al., 1977). Here, the dominating sequence will light up as the consensus sequence during sequencing. The minor variants will remain obscured by the master's sequencing signals. In contrast, when using next generation sequencing methods, the consensus is built by special software (for instance the assembler software newbler accompanying the Genome Sequencer FLX [454 Life Sciences, 2009]) from individual sequencing reads that reflect individual input molecules. As pointed out already, the consensus sequence reflects the master sequence while retaining information about minor variants in the respective reads. Hence, only with the advent of the different next generation sequencing technologies the examination of genetically diverse populations became efficiently feasible for the first time. Therefore, another aim is to elucidate whether or not lyssaviruses comprise a quasi-species and if so to analyse the extent and impact of their quasi-species composition.

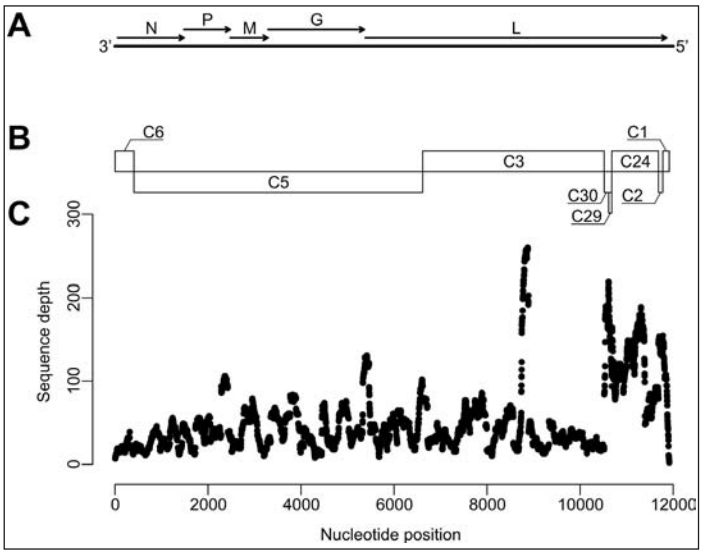

FIGURE 1: *Overview of the CVS-11 genome reference sequence and shotgun sequencing assembly. (A) Full-length reference genome depicted as a horizontal line together with arrows representing the genes encoding the nucleoprotein (N), the phosphoprotein (P), the matrixprotein (M), the glycoprotein (G), and the viral RNA-dependent RNA-polymerase (L). (B) Combination of the contigs (each contig labelled with prefix "C" and its number) from the assembly of the shotgun sequencing data yielding the full-length genome sequence. (C) Depth of the sequence depicted in panel B.*

For several viral species, amongst these also some lyssavirus species, another means of diversification of their genome is the generation of so called defective interfering (DI) particles (Lazzarini et al., 1981; Perrault, 1981). Lyssaviruses are long known to generate DI particles (Crick and Brown, 1974; Wiktor et al., 1977). These are characterized by the sub-genomic nucleic acids they comprise. While for vesicular stomatitis virus (VSV) it was shown that DI RNAs equipped with the genomic 5′-terminus on both ends exist (Rao and Huang, 1982), for lyssaviruses to date only DI RNAs with internal deletions have been described. Compared to the native genomes, DI RNAs with the stronger anti genome promoter from the genomic 5′-terminus at both ends are preferentially replicated (Calain and Roux, 1995) and are efficiently packaged into virions. Therefore, the number of DI RNAs that is becoming assembled into virions may exceed the number of fully functional full-length genomes assembled into virions. Due to their incomplete genomes, the DI particles rely on co-infection with particles containing full genomes to provide the proteins that are necessary for virus replication and assembly (Lazzarini et al., 1981). Conzelmann and co-workers (1991) analysed RNA from DI particles by hybridisation experiments and partial sequencing to locate the internal deletions of the DI RNA. Besides these analyses there is only little knowledge of the sequences of lyssavirus DI RNAs available. Until now, although a few sequences from viral DI particles are available, no sequences from lyssavirus DI RNA can be found in the sequence databases (e. g. Genbank). This lack of knowledge may be caused by the complications in sequence analysis that are implicated in Sanger sequencing of heterogeneous populations of nucleic acids.

Also with regard to the implications of genome diversity for pathogenicity next to nothing is known. In

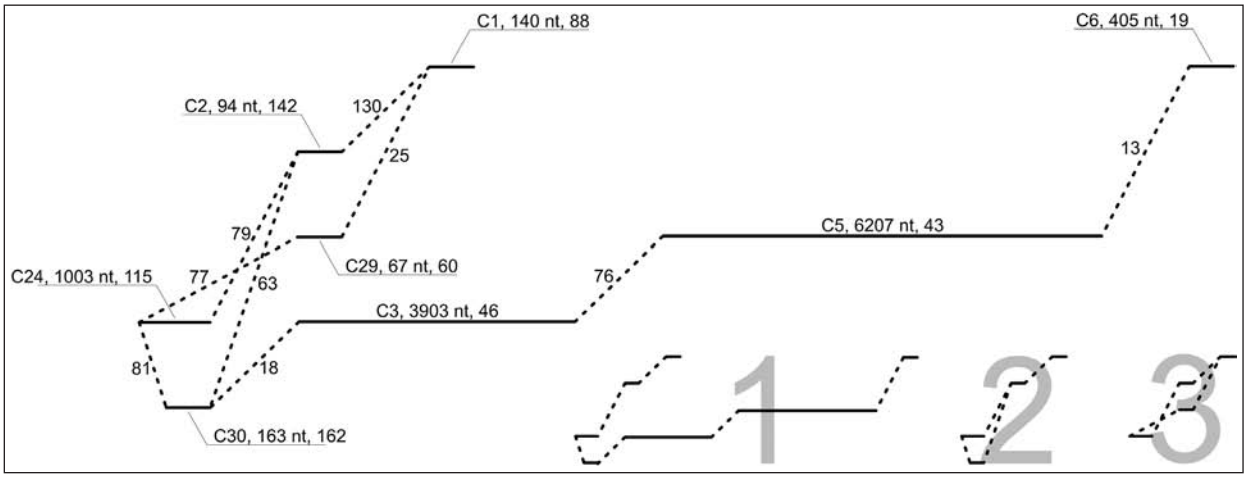

FIGURE 2: *Graphical representation of the contig interconnections as provided by the assembler. Horizontal lines depict the contigs the software built from the raw data; dashed lines are connections that were detected between the contigs during the assembly. In the large display, every contig is labelled with its identifier (a number with the prefix "C" for contig), followed by the length and the mean depth of the contig. The dashed contig connectors are labelled with the number of individual sequence reads that establish the connection. Note that contigs of less than 620 bases are not true to scale. The small numbered displays show the three different ways through the graph that are possible.*

general, the quasi-species is assumed to be the basis for adaptability of the virus. Therefore, analysis of it may provide insight into the virus-host relation and the evolution of pathogenicity. On the contrary, the relevance of DI particles for pathogenicity remains speculative. There is evidence that only with the aid of DI particles VSV is able to establish a persistent infection in cell culture (Holland and Perez Villarreal, 1974). Also for rabies virus it was shown that DI particles are present at all time in persistently infected cell cultures (Grabau and Holland, 1982). On the basis of these data it is tempting to speculate that lyssaviruses may even establish a permanent infection without clinical signs in their reservoir species. Fitting this speculation, Rieder and Conzelmann (2011) recently hypothesized that rabies virus DI RNA could be accessible by RIG-I and thus could modulate the interferon (IFN) response in the host. A correlation between IFN induction and DI RNA levels was shown for paramyxoviruses and VSV (Strahle et al., 2006; Panda et al., 2010). Thus, knowledge about DI particles both with regard to quality, i. e. the different sequence variants, and quantity of the respective variants may also help devise and prove hypothesis about the possible influence of lyssavirus DI particles on pathogenicity. Therefore, we set out to start analysing the diversity of the lyssavirus genomes in order to find out whether or not there is a diverse population at all and, if so, to determine the diversity and analyse the impact of the population on lyssavirus biology.

First results and perspectives

Both the quasi-species analysis and the analysis of the structure of defective interfering genomes are becoming feasible by the recent advances in DNA sequencing technologies. This is due to the overwhelming sequence depth (the sequence depth is the number of times each base was sequenced) that can be achieved and the possibility to randomly start sequencing of DNA/RNA to detect structural variations. For instance, Leifer and colleagues (2010) used the considerable sequence depth they achieved using the Genome Sequencer FLX to analyse the diversity of the viral population. They were able to draw conclusions about the evolutionary relations of different classical swine fever virus isolates. Also for Influenza A H5N1 viruses the analysis of the viral population in relation to its evolution during prolonged passaging was possible (Höper et al., 2012).

Whereas quasi-species analysis was already shown to be feasible with next generation sequencing data, until now these data were not analysed with respect to viral structural genome variants. Therefore, in a pilot experiment, the genome of rabies virus strain CVS-11 cultured in murine neuroblastoma cells was sequenced on a Roche/454 Genome Sequencer FLX. The strain

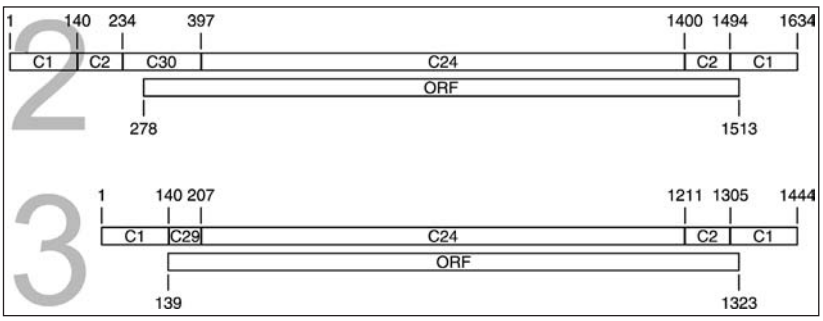

FIGURE 3: *Linear depiction of variants two and three that can be built from the contigs and their possible connections as displayed in figure 1. Every contig is represented by a box labelled with its number with the prefix "C" for contig. In addition, the open reading frames that were detected in the combined sequences are also depicted as a box labelled "ORF". Numbers above and underneath the depiction of contigs and ORFs mark nucleotide positions of contig junctions and start and end of the open reading frames, respectively.*

TABLE 1: *Overview of the genome variants that can be constructed from the sequence contigs built by the* 454 assembler software newbler

Variant	Contigs included	Length	Remarks
1	6, 5, 3, 30, 24, 2, 1	11915	full genome
2	1, 2, 30, 24, 2, 1	1634	DI RNA with genomic 5'-terminus on both ends; ORF from nt 278–1513; codes RNA dependent RNA polymerase AA 1717–2127
3	1, 29, 24, 2, 1	1444	DI RNA with genomic 5'-terminus on both ends; ORF from nt 139–1323; codes RNA dependent RNA polymerase AA 1734–2127

CVS-11 was chosen for this pilot experiment as it is a well characterized prototypic lyssavirus, for instance with regard to pathogenicity. Moreover, for this strain a reference genome sequence exists which enables the assessment of the general suitability of the sequencing technique. For preparation of the sequencing library, cDNA synthesis was randomly primed anticipating to evenly convert the complete population of virus genomes into cDNA. This should, on one hand, enable quasi-species analysis, i. e. detection of single nucleotide polymorphisms present in the population. On the other hand, the sequencing data should in addition provide information on structural reorganisation of the genomes as in the case of defective interfering RNAs. Therefore, to yield high amounts of viral sequences, the RNA used for this experiment was extracted from virions that were purified by sucrose gradient centrifugation. Sequencing of the resultant library yielded approximately 5500 reads with roughly 730 000 bases of raw lyssavirus sequence, i. e. a 60-fold oversampling of the genome. The raw data obtained in this pilot study were analysed with respect to the quasi-species composition and the structural variants that were part of the virus population. Despite the enormous amount of raw data, the assembly ended up with fragmented genome sequences instead of a single contig representing the full-length genome one would expect (Fig. 1, panels B and C). During sequence assembly, fragmentation of the sequences may be caused by nucleotide positions where there is no clearly dominating base. At such positions, the assembler is not able to determine the consensus base. This may cause low quality basecalls, single base insertions or deletions, or the sequence is split into multiple contigs at these ambiguous positions. In addition to the contig sequences and the base qualities, the assembler software newbler provides information about the detected interconnections of all the contigs that were built during the assembly. A graphical representation of this information for the sequenced rabies virus strain CVS-11 is provided in Figure 2. Making use of this information, it was possible to establish the (nearly) full-length genome sequence (Fig. 1, panel B and variant one in Fig. 2). In addition, as can also be seen in Figure 2, the interconnections that were detected by the software provide a hint that different sub-genomic nucleic acids were purified from the virions. In total, three different variant sequences could be constructed from the contigs making use of the information about contig interconnections. The main properties of these variant sequences are summarized in Table 1. In order to assess the quality and significance of these alternative contig connections, the sequences were combined accordingly and subsequently analysed in silico. Variant number one constitutes the complete genome (Fig. 1B). The quality of the full-length genome sequence was assessed by comparison of the newly established with the sequence present in the database. Only a few single base exchanges were detected. These variations are probably caused by different histories of the viruses that were sequenced to get the reference or the NGS shotgun sequence, respectively. Therefore, one can assume that the random sequencing approach yields reliable sequence information. The other two sequence variants, variants two and three (Fig. 2), represent most likely defective interfering RNAs. They have the stronger anti genome promoter at both ends and only comprise a short portion of the L-protein gene. Analyses of the sequences revealed that these defective interfering RNAs code for truncated versions of the RNA-dependent RNA-polymerase. In both cases the open reading frames extend across the contig junctions (Fig. 3). Translation of the open reading frames into amino acid sequences results in truncated RNA-dependent RNA polymerase sequences perfectly matching the C-terminal part (amino acids 1717–2127 and amino acids 1734–2127, variant number two and three, respectively) of the original CVS-11 polymerase protein. We neither expect that gene expression from these DI RNAs might occur nor do we have any hints implying that this might happen. Nevertheless, this extension of the open reading frame across the fusion site lets it seem unlikely that this fusion is a randomly occurring sequencing artefact. Even though these findings seem not very likely to be artificially introduced, they need to be experimentally proven. To provide this proof will also be part of this project in the future. In addition to the sequences, the software together with the information about contig interconnections provides the mean depth of the contigs and the number of reads that constitute the connections. From this information that is also included in Figure 2, it can be seen that the sequence depth of the contigs that are part of presumable defective interfering RNAs is significantly higher than that of the other contigs. Additionally, the number of interconnections of the contigs that are included in the defective interfering sequences is higher than those of the other contigs. The higher share of the contigs included in defective interfering RNAs over those that are only part of the complete genome fits the higher frequencies that are expected for defective interfering RNAs.

The aforementioned results emphasize for the first time the suitability of the Genome Sequencer FLX technology to get high quality full-length genome sequences of lyssaviruses on the one hand and on the other hand detailed information about structural variants that are part of the in vitro released virus population. Despite the lack of direct experimental proof for the structural variation that was found and the fact that the biological impact of this structural variations remains to be further elucidated, the presented findings highlight the power of the sequencing technology to help gain novel knowledge of the structural variability and evolution of viral genomes in general and of lyssavirus genomes in particular. The investigated CVS-11 is a prototypic lyssavirus with known properties in terms of pathogenicity after inoculation in mice, for instance. Certainly, other rabies virus strains and other lyssavirus species,

for example European bat lyssavirus 2, have differing pathogenicity (Vos et al., 2004; Johnson et al., 2008). By comparative analysis of their quasi-species and structural variants, the role of DI RNAs in the activation of IFN and thus in the pathogenicity will be elucidated.

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