Distinct roles for the IIId2 sub-domain in pestivirus and picornavirus internal ribosome entry sites

Viral internal ribosomes entry site (IRES) elements coordinate the recruitment of the host translation machinery to direct the initiation of viral protein synthesis. Within hepatitis C virus (HCV)-like IRES elements, the sub-domain IIId(1) is crucial for recruiting the 40S ribosomal subunit. However, some HCV-like IRES elements possess an additional sub-domain, termed IIId2, whose function remains unclear. Herein, we show that IIId2 sub-domains from divergent viruses have different functions. The IIId2 sub-domain present in Seneca valley virus (SVV), a picornavirus, is dispensable for IRES activity, while the IIId2 sub-domains of two pestiviruses, classical swine fever virus (CSFV) and border disease virus (BDV), are required for 80S ribosomes assembly and IRES activity. Unlike in SVV, the deletion of IIId2 from the CSFV and BDV IRES elements impairs initiation of translation by inhibiting the assembly of 80S ribosomes. Consequently, this negatively affects the replication of CSFV and BDV. Finally, we show that the SVV IIId2 sub-domain is required for efficient viral RNA synthesis and growth of SVV, but not for IRES function. This study sheds light on the molecular evolution of viruses by clearly demonstrating that conserved RNA structures, within distantly related RNA viruses, have acquired different roles in the virus life cycles.
Evolutionary analysis of whole-genome sequences confirms inter-farm transmission of Aleutian mink disease virus

Aleutian mink disease virus (AMDV) is a frequently encountered pathogen associated with mink farming. Previous phylogenetic analyses of AMDV have been based on shorter and more conserved parts of the genome, e.g. the partial NS1 gene. Such fragments are suitable for detection but are less useful for elucidating transmission pathways while sequencing entire viral genomes provides additional informative sites and often results in better-resolved phylogenies. We explore how whole-genome sequencing can benefit investigations of AMDV transmission by reconstructing the relationships between AMDV field samples from a Danish outbreak. We show that whole-genome phylogenies are much better resolved than those based on the partial NS1 gene sequences extracted from the same alignment. Well-resolved phylogenies contain more information about the underlying transmission trees and are useful for understanding the spread of a pathogen. In the main case investigated here, the transmission path suggested by the tree structure was supported by epidemiological data. The use of molecular clock models further improved tree resolution and provided time estimates for the viral ancestors consistent with the proposed direction of spread. It was however impossible to infer transmission pathways from the partial NS1 gene tree, since all samples from the case farms branched out from a single internal node. A sliding window analysis showed that there were no shorter genomic regions providing the same phylogenetic resolution as the entire genome. Altogether, these results suggest that phylogenetic analyses based on whole-genome sequencing taking into account sampling dates and epidemiological data is a promising set of tools for clarifying AMDV transmission.

General information
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Organisations: Molecular Evolution, Department of Biotechnology and Biomedicine, Department of Bio and Health Informatics, Disease Intelligence and Molecular Evolution, National Veterinary Institute, Virology, Kopenhagen Fur
Authors: Hagberg, E. E. (Intern), Pedersen, A. G. (Intern), Larsen, L. E. (Intern), Krarup, A. (Ekstern)
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A fast and robust method for whole genome sequencing of the Aleutian Mink Disease Virus (AMDV) genome

Aleutian Mink Disease Virus (AMDV) is a frequently encountered pathogen associated with commercial mink breeding. AMDV infection leads to increased mortality and compromised animal health and welfare. Currently little is known about the molecular evolution of the virus, and the few existing studies have focused on limited regions of the viral genome. This paper describes a robust, reliable, and fast protocol for amplification of the full AMDV genome using long-range PCR. The method was used to generate next generation sequencing data for the non-virulent cell-culture adapted AMDV-G strain as well as for the virulent AMDV-Utah strain. Comparisons at nucleotide- and amino acid level showed that, in agreement with existing literature, the highest variability between the two virus strains was found in the left open reading frame, which encodes the non-structural (NS1–3) genes. This paper also reports a number of differences that potentially can be linked to virulence and host range. To the authors’ knowledge, this is the first study to apply next generation sequencing on the entire AMDV genome. The results from the study will facilitate the development of new diagnostic tools and can form the basis for more detailed molecular epidemiological analyses of the virus.

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Outbreaks of Aleutian mink disease in farmed mink (Neovison vison) in Denmark: molecular characterization by partial NS1 gene sequencing

Using expected sequence features to improve basecalling accuracy of amplicon pyrosequencing data

Using expected sequence features to improve basecalling accuracy of amplicon pyrosequencing data obtained from a malaria virulence gene family, where Multipass generates 20 % more error-free sequences than current state of the art methods, and provides sequence characteristics that allow generation of a set of high confidence error-free sequences. This novel method can be used to increase accuracy of existing and future amplicon sequencing data, particularly where extensive prior knowledge is available about the obtained sequences, for example in analysis of the immunoglobulin VDJ region where Multipass can be combined with a model for the known recombining germline genes. Multipass is available for Roche 454 data at http://www.cbs.dtu.dk/services/MultiPass-1.0, and the concept can potentially be implemented for other sequencing technologies as well.
RNA viruses have the highest known mutation rates. Consequently it is likely that a high proportion of individual RNA virus genomes, isolated from an infected host, will contain lethal mutations and be non-functional. This is problematic if the aim is to clone and investigate high-fitness, functional cDNAs and may also pose problems for sequence-based analysis of viral evolution. To address these challenges we have performed a study of the evolution of classical swine fever virus (CSFV) using deep sequencing and analysis of 84 full-length cDNA clones, each representing individual genomes from a moderately virulent isolate. In addition to here being used as a model for RNA viruses generally, CSFV has high socioeconomic importance and remains a threat to animal welfare and pig production. We find that the majority of the investigated genomes are non-functional and only 12% produced infectious RNA transcripts. Full length sequencing of cDNA clones and deep sequencing of the parental population identified substitutions important for the observed phenotypes. The investigated cDNA clones were furthermore used as the basis for inferring the sequence of functional viruses. Since each unique clone must necessarily be the descendant of a functional ancestor, we hypothesized that it should be possible to produce functional clones by reconstructing ancestral sequences. To test this we used phylogenetic methods to infer two ancestral sequences, which were then reconstructed as cDNA clones. Viruses rescued from the reconstructed cDNAs were tested in cell culture and pigs. Both reconstructed ancestral genomes proved functional, and displayed distinct phenotypes in vitro and in vivo. We suggest that reconstruction of ancestral viruses is a useful tool for experimental and computational investigations of virulence and viral evolution. Importantly, ancestral reconstruction can be done even on the basis of a set of sequences that all correspond to non-functional variants.

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Organisations: National Veterinary Institute, Section for Virology, Department of Systems Biology, Center for Biological Sequence Analysis, Molecular Evolution, Friedrich Loeffler Institute, University of Glasgow
Authors: Fahnøe, U. (Intern), Pedersen, A. G. (Intern), Dräger, C. (Ekstern), Orton, R. J. (Ekstern), Blome, S. (Ekstern), Höper, D. (Ekstern), Beer, M. (Ekstern), Rasmussen, T. B. (Intern)
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Early Divergent Strains of *Yersinia pestis* in Eurasia 5,000 Years Ago

The bacteria *Yersinia pestis* is the etiological agent of plague and has caused human pandemics with millions of deaths in historic times. How and when it originated remains contentious. Here, we report the oldest direct evidence of *Yersinia pestis* identified by ancient DNA in human teeth from Asia and Europe dating from 2,800 to 5,000 years ago. By sequencing the genomes, we find that these ancient plague strains are basal to all known *Yersinia pestis*. We find the origins of the *Yersinia pestis* lineage to be at least two times older than previous estimates. We also identify a temporal sequence of genetic changes that lead to increased virulence and the emergence of the bubonic plague. Our results show that plague infection was endemic in the human populations of Eurasia at least 3,000 years before any historical recordings of pandemics.

General information

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Organisations: Department of Systems Biology, Center for Biological Sequence Analysis, Metagenomics, Molecular Evolution, Eucaryotic Molecular Cell Biology, Integrative Systems Biology, University of Copenhagen, National Academy of Sciences of the Republic of Armenia, South Ural State University, Orenburg Museum of Fine Arts, Yerevan State University, University of Tartu, University of Wroclaw, Peter the Great Museum of Anthropology and Ethnography,
Complete Genome Sequence of Border Disease Virus Genotype 3 Strain Gifhorn
The complete genome sequence of the genotype 3 border disease virus strain Gifhorn has been determined; this strain was originally isolated from pigs. This represents the consensus sequence for the virus used to produce the bacterial artificial chromosome (BAC) cDNA clone pBeloGif3, which yields a virus that is severely attenuated in cell culture.

Complete Genome Sequence of Classical Swine Fever Virus Genotype 2.2 Strain Bergen
The complete genome sequence of the genotype 2.2 classical swine fever virus strain Bergen has been determined; this strain was originally isolated from persistently infected domestic pigs in the Netherlands and is characterized to be of low virulence.
Rescue of the highly virulent classical swine fever virus strain “Koslov” from cloned cDNA and first insights into genome variations relevant for virulence

Classical swine fever virus (CSFV) strain “Koslov” is highly virulent with a mortality rate of up to 100% in pigs. In this study, we modified non-functional cDNAs generated from the blood of Koslov virus infected pigs by site-directed mutagenesis, removing non-synonymous mutations step-by-step, thereby producing genomes encoding the consensus amino acid sequence. Viruses rescued from the construct corresponding to the inferred parental form were highly virulent, when tested in pigs, with infected animals displaying pronounced clinical symptoms leading to high mortality. The reconstruction therefore gave rise to a functional cDNA corresponding to the highly virulent Koslov strain of CSFV. It could be demonstrated that two single amino acid changes (S763L and P968H) in the surface structural protein E2 resulted in attenuation in the porcine infection system while another single amino acid change within the nonstructural protein NS3 (D2183G) reduced virus growth within cells in vitro.
Studies on genetic diversity of bovine viral diarrhea viruses in Danish cattle herds

Scandinavian countries have successfully pursued bovine viral diarrhea virus (BVDV) eradication without the use of vaccines. In Denmark, control and eradication of BVDV were achieved during the last two decades, but occasionally new BVDV infections are detected in some Danish cattle herds. The aim of this study was to determine recent BVDV subtypes isolated from 4 Danish herds (A, B, C, and D) isolated in 2009–2012 and to analyze the genetic variation of these isolates within the same herd and its relation with those of other herds. The results showed that three herds (B, C, D) were BVDV 1-b and only one herd (herd A) was BVDV 1-d, no other subtypes were detected. The deduced E2 amino acids result showed a high identity percent (99–100 %) between isolates originating from the same herd, but with higher variation
compared to isolates of the other herds. Some of these new Danish strains have closer relationship to BVDVs from outside Denmark than to older Danish strains indicating that these are new introductions to Denmark. In conclusion, BVDV-1 subtypes recently detected in Denmark were only subtypes 1b and 1d, and BVDV infections established in a herd is genetically stable over a long time period.

**General information**

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Organisations: National Veterinary Institute, Section for Virology, Molecular Evolution, Technical University of Denmark
Authors: Nagy, A. (Ekstern), Fahnøe, U. (Intern), Rasmussen, T. B. (Intern), Uttenthal, Å. (Intern)
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Scopus rating (2012): SJR 0.755 SNIP 0.809 CiteScore 1.8
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Scopus rating (2011): SJR 0.834 SNIP 0.996 CiteScore 1.92
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Scopus rating (2006): SJR 0.64 SNIP 0.566
Scopus rating (2005): SJR 0.646 SNIP 0.622
Web of Science (2005): Indexed yes
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Scopus rating (2002): SJR 0.569 SNIP 0.524
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Targeting the genetic complexity within adapting RNA virus populations

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Authors: Fahnøe, U. (Intern), Pedersen, A. G. (Intern), Rasmussen, T. B. (Intern)
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A fast and robust method for full genome sequencing of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Type 1 and Type 2

PRRSV is a positive-sense RNA virus with a high degree of genetic variability among isolates. For diagnostic sensitivity and vaccine design it is essential to monitor PRRSV genetic diversity. However, to date only a few full genome sequences of PRRSV isolates have been made publicly available. In the present study, fast and robust methods for long range RT-PCR amplification and subsequent next generation sequencing (NGS) were developed and validated on nine Type 1 and nine Type 2 PRRSV viruses. The methods generated robust and reliable sequences both on primary material and cell culture adapted viruses and the protocols performed well on all three NGS platforms tested (Roche 454 FLX, Illumina HiSeq2000, and Ion Torrent PGM™ Sequencer). These methods will greatly facilitate the generation of more full genome PRRSV sequences globally.

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Organisations: National Veterinary Institute, Section for Virology, Section for Public sector service and commercial diagnostics, Molecular Evolution, University of Edinburgh
Authors: Kvisgaard, L. K. (Intern), Hjulsager, C. K. (Intern), Fahnøe, U. (Intern), Breum, S. Ø. (Intern), Ait-Ali, T. (Ekstern), Larsen, L. E. (Intern)
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Comparison of two Next Generation sequencing platforms for full genome sequencing of Classical Swine Fever Virus

Next Generation Sequencing (NGS) is becoming more adopted into viral research and will be the preferred technology in the years to come. We have recently sequenced several strains of Classical Swine Fever Virus (CSFV) by NGS on both Genome Sequencer FLX (GS FLX) and Iontorrent PGM platforms. In this study, we analyzed NGS data of virus rescued from a CSFV C-strain vaccine strain cDNA clone. The virus analyzed was obtained from a 4th and a 12th passage of rescued virus in SFT cell culture, which had shown a difference in growth kinetics between the passages, and NGS analysis was chosen in order to look for molecular differences. Identical RT-PCR products were run on both GS FLX and Iontorrent PGM platform for comparison. The NGS data was compared by quality and the percentage mapped reads. Results showed good quality of reads for both platforms and a close to 100% of the reads mapped to the consensus
Efficient generation of recombinant RNA viruses using targeted recombination-mediated mutagenesis of bacterial artificial chromosomes containing full-length cDNA

Background
Infectious cDNA clones are a prerequisite for directed genetic manipulation of RNA viruses. Here, a strategy to facilitate manipulation and rescue of classical swine fever viruses (CSFVs) from full-length cDNAs present within bacterial artificial chromosomes (BACs) is described. This strategy allows manipulation of viral cDNA by targeted recombination-mediated mutagenesis within bacteria.

Results
A new CSFV-BAC (pBeloR26) derived from the Riems vaccine strain has been constructed and subsequently modified in the E2 coding sequence, using the targeted recombination strategy to enable rescue of chimeric pestiviruses (vR26_E2gif and vR26_TAV) with potential as new marker vaccine candidates. Sequencing of the BACs revealed a high genetic stability during passages within bacteria. The complete genome sequences of rescued viruses, after extensive passages in mammalian cells showed that modifications in the E2 protein coding sequence were stably maintained. A single amino acid substitution (D3431G) in the RNA dependent RNA polymerase was observed in the rescued viruses vR26_E2gif and vR26, which was reversion to the parental Riems sequence.

Conclusions
These results show that targeted recombination-mediated mutagenesis provides a powerful tool for expediting the construction of novel RNA genomes and should be applicable to the manipulation of other RNA viruses.
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Genetic and antigenic characterization of influenza A virus circulating in Danish swine during the past decade

Influenza A virus has been endemic in Danish swine for the last 30 years, with H1N1 and H1N2 being the dominating subtypes. The purpose of this study was to investigate the genetic and antigenic evolution of the influenza viruses found in Danish swine during the last 10 years. A total of 78 samples were isolated in MDCK cells, RNA extracted and the hemagglutinin and neuraminidase genes full length sequenced. In addition, the isolates were tested in hemagglutination inhibition (HI) tests against a panel of known antisera raised against a range of European swine influenza virus isolates. Phylogenetic analysis of the HA and NA genes revealed continuous evolutionary drift as expected for RNA viruses with low mutational selection pressure. Estimated selection pressures indicated that more purifying and less diversifying selection controlled the H1 evolution. The mean rates of synonymous and non-synonymous substitutions for H1, N1 and N2 were found to be in agreement with previously observed values for Eurasian swine lineages. Calculation of possible glycosylation sites in the hemagglutinin gene revealed that the H1N2 and H1N1 subtypes had three well conserved glycosylation sites in common. The results of the HI tests were analysed by antigenic cartography to quantify the antigenic relationship between the virus isolates. The antigenic cartography map showed that most of the Danish viruses were antigenic very similar, with only a few outliers. In conclusion, this study provided an important contribution to the complex epidemiology of circulating swine influenza virus in Denmark and indicates that vaccine development targeted against Danish H1N1 and H1N2 need only to include few components for the induction of cross protection against the predominant strains.

The study was supported by grants from “European surveillance network for influenza in pigs (ESNIP) 3” (http://www.esnip3.eu) and The Danish Veterinary and Food Administration.

Reconstructing the highly virulent Classical Swine Fever Virus strain Koslov

Classical swine fever virus (CSFV) may be highly virulent in pigs with a mortality rate close to 100%. The CSFV “Koslov strain” is known to be one of the most virulent CSFV, but so far a functional cloned cDNA of this strain has not been described. We suggest that this may be due to the error-prone nature of the RNA-dependent RNA polymerase resulting in the majority of circulating forms being non-functional. However, since any infectious virus particle should necessarily be the offspring of a functional virus, we hypothesized that it should be possible to synthesize a highly virulent form by reconstructing ancestral sequences. To test this hypothesis, we inferred sequences that correspond to ancestral nodes in a phylogenetic tree built from full-length nucleotide sequences of non-functional Koslov cDNAs and then proceeded to test the reconstructions. Specifically, we altered a non-functional cDNA by site directed mutagenesis, removing non-synonymous mutations step by step. In vitro testing of modified constructs did indeed lead to fully functional viruses with similar growth kinetics as the wild-type strain. Moreover, viruses rescued from the construct had the ancestral amino acid sequence and, when tested in pigs, were at least as virulent as the Koslov strain. The ancestral reconstruction therefore proved to give rise to a functional cDNA of the highly virulent Koslov strain. In vivo studies confirmed our methods and enabled us to identify nucleotide positions within the viral genome important for virulence.

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Organisations: National Veterinary Institute, Section for Virology, Department of Systems Biology, Integrative Systems Biology, Molecular Evolution, Section for Public sector service and commercial diagnostics, University of Cambridge
Authors: Fobian, K. (Intern), Kirk, I. K. (Intern), Breum, S. Ø. (Intern), Lewis, N. (Ekstern), Hjulsager, C. K. (Intern), Larsen, L. E. (Intern)
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