Sequence-independent VIDISCA-454 technique to discover new viruses in canine livers

Mitzi van der Heijden, Michel de Vries, Frank G. van Steenbeek, Robert P. Favier, Martin Deijs, Bas Brinkhof, Jan Rothuizen, Lia van der Hoek, Louis C. Penning

Article history:
Received 22 July 2011
Received in revised form 15 May 2012
Accepted 24 May 2012
Available online 1 June 2012

Keywords:
Sequence-independent Virus Discovery VIDISCA
High throughput sequencing
Viruses
Dogs

A B S T R A C T

In many mammals, viruses cause hepatitis. Despite many efforts a specific virus responsible for canine idiopathic hepatitis has not been identified. The discovery of a viral etiology in canine hepatitis will promote the development of specific drugs and vaccines for the treatment of idiopathic hepatitis in dogs. The objective of this study was the application of the sequence-independent Virus Discovery cDNA-amplified fragment length polymorphism (VIDISCA) technique combined with high through-put sequencing on a Roche-454 sequencer to identify unknown viruses. Liver tissue of a dog with idiopathic acute hepatitis was cultured on a canine liver cell line and the cell culture medium was submitted to the VIDISCA-454 technique. Without prior knowledge of the viral species involved, this technique identified Canine adenovirus type 1 (CAV-1) as the infecting agent. This demonstrates the power of VIDISCA-454 to identify viruses, independent of preliminary information about the genomic sequence. Consequently, the strategy of propagation in this cell line followed by the VIDISCA-454 technique is valuable to identify the viral etiology of idiopathic hepatitis in dogs.

Primary hepatitis is probably the most frequent liver disease in dogs with a reported prevalence in the general dog population of about 12% (Watson et al., 2010). Primary hepatitis comprises inflammatory hepatic diseases excluding non-specific reactive hepatitis (Poldervaart et al., 2009). The diagnosis of hepatitis in dogs is based on histological morphology, and the term is often used regardless of the etiology. The World Small Animal Veterinary Association (WSAVA) Liver Standardization Group has published standards for the diagnosis (Van den Ingh et al., 2006). Various microbiological-, toxin-, drug- and immune-mediated causes have been documented (Decaro et al., 2008; Newman et al., 2007; Watson, 2004; Tweedt et al., 1997; Fox et al., 1996; Weiss et al., 1995). Recently, a survey estimated that around 30% of canine hepatitis was related to copper accumulation in a Dutch population referred to the third-line university clinic (Poldervaart et al., 2009).

Yet, about two third of the dogs with hepatitis have an unknown etiology.

A viral etiology for idiopathic hepatitis has been proposed for decades. Despite many efforts made, a specific virus responsible for canine idiopathic hepatitis has not been identified (Boomkens et al., 2005; Watson, 2004; Jarrett et al., 1987; Jarrett and O’Neil, 1985; Gocke et al., 1967). Highly sensitive PCR with (degenerated) primers based on known viruses failed thus far, emphasizing the need to develop viral discovery strategies that can detect viruses without any knowledge of the genomic sequences (Bexfield and Kellam, 2011).

One of the recent developments in sequence-independent approaches for the discovery of unknown viruses is the Virus Discovery cDNA-amplified fragment length polymorphism (VIDISCA) technique (van der Hoek et al., 2004). It is based on a sequence-independent amplification of cDNA from test and control samples using restriction enzymes and the ligation of oligonucleotide adapters containing primer annealing sites (Engel et al., 2010; de Souza Luna et al., 2008; Pyrc et al., 2008; Ambrose and Clewley, 2006; van der Hoek et al., 2004). However, as a consequence of the susceptibility for interfering nucleotides (e.g. host ribosomal RNA and chromosomal DNA), this technique is only suitable for samples, which contain a minimal amount of background-sequences or samples with a high viral load (van der Hoek et al., 2004). Nevertheless, this problem is overcome by combining the VIDISCA technique.
with high through-put next generation sequencing. Roche-based 454 next generation sequencing can sequence 800,000–1,000,000 fragments with an average read length of 400–500 nucleotides in a single run (Roche, Basel, Switzerland). The combination of VIDISCA with 454 sequencing allows efficient and sensitive virus discovery in respiratory material (de Vries et al., 2011).

In the present study the feasibility is described of VIDISCA-454 to obtain viral sequence information in an idiopathic canine hepatitis liver biopsy. To increase the viral load, a canine liver cell line (BDE, bile duct epithelial) (Spee et al., 2008) was subjected to a homogenate of liver samples from a dog with idiopathic hepatitis.

Post-mortem liver samples were used from a four year old mixed breed dog, referred to the Department of Clinical Sciences of Companion Animals of the Veterinary faculty (Utrecht University, The Netherlands), that had died because of acute hepatitis, present clinically for four days. Liver tissue was snap frozen in liquid N$_2$ and stored in the freezer at −70 °C. Histopathological evaluation according to WSAVA world standards for liver histology (Van den Ingh et al., 2006) by a Board-certified pathologist established the diagnosis of severe acute idiopathic hepatitis. Canine bile duct epithelial (BDE) cells, an established cell line for dog hepatocytes, were cultured as described by Spee et al. (2008). Liver tissue (wedge biopsy −125 mm$^3$) from the affected dog was homogenized in cell culture medium (DMEM: high glucose, supplemented with 10% fetal calf serum (FCS) and 1% Penicillin (100 U/ml)/Streptomycin (100 µg/ml)) using a pestle, vortexed and then centrifuged (Eppendorf® Centrifuge 4515; 3300 × g, 5 min) to pellet cellular debris. BDE cells were inoculated with the supernatant. The cells were monitored daily by light microscopy for cytopathogenic effect. After 5 days the cell culture medium was transferred to Eppendorf® tubes and the cell culture plates were subjected to a three times repeated snap freeze/thaw procedure to disrupt the cellular membranes and release the viral particles. New cultured BDE cells were inoculated with this freeze/thaw suspension (second inoculation). Five days later this procedure was repeated (third inoculation).

Additionally, during the cell culture experiment the culture medium was collected three days after each inoculation and following centrifugation (Eppendorf® Centrifuge 4515; 200 × g, 5 min) the supernatant was stored at −70 °C. Subsequently fresh cell culture medium was added to the BDE cells. Four days after the third inoculation the experiment was ended and supernatant of the culture medium was collected and treated as above. This experiment was performed twice in duplicate, in order to assess the reproducibility. Duplicates of the culture supernatant from both cell culture experiments were submitted separately (Exp1 and Exp2, respectively) to the VIDISCA–454 high through-put sequencing technique.

To purify viral particles, 110 µl of culture supernatant was centrifuged (10,000 × g, 12 min) and treated with 20 U of Turbo DNase (Ambion, Nieuwekerk a/d IJssel, The Netherlands). After inactivation of DNase, the viral capsids were degraded and their nuclear material was isolated. cDNA was synthesized using superscript II (Invitrogen, Breda, The Netherlands). A mixture of 96 specific non-ribosomal hexanucleotides was used in the reverse transcriptase reaction to minimize the amount of ribosomal RNA converted to cDNA (Endoh et al., 2005). The second strand was synthesized with Klenow exo-polymerase (New England Biolabs, Westburg, Leusden, The Netherlands) and a phenol–chloroform extraction was followed by ethanol precipitation.

Virus Discovery cDNA-amplified fragment length polymorphism (VIDISCA) in combination with 454-high throughput sequencing was performed as described (de Vries et al., 2011). The double stranded DNA was digested with Msel restriction enzyme (New England Biolabs, Westburg, Leusden, The Netherlands). After digestion of the DNA, specific A and B-adapters (Roche) were ligated to the DNA fragments. Both adapters contain a primer-binding site for the sequence independent amplification. The B-adaptor, containing a capture bead annealing site necessary for the 454-sequencing reaction, is similar for all samples. The A-adaptor however, is unique for each sample and contains a multiplex identifier (MID), which is linked to the sample concerned. MID7 was assigned to the culture supernatant of Exp1 and MID8 was assigned to the culture supernatant of Exp2 (adapter sequences are depicted in Table 1). After ligation (Invitrogen) of the adapters the DNA fragments were amplified in a polymerase chain reaction (PCR) (AmpliTaq polymerase, Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands).

The PCR products were analyzed on 1% agarose gels and amplicons between 200–300 bp, 300–500 bp and 500–700 bp were collected and submitted separately to gel extraction (Nucleospin® Extract II). The DNA concentration of the extractions was measured using the Qubit® Quantitation Platform (Invitrogen) and the number of DNA copies was calculated. All samples were diluted 1.00 × 10$^7$ copies/µl before continuing with the 454-protocol. A bead bound DNA library was prepared, followed by clonal amplification of the bead bound DNA fragments during an emulsion PCR. After the amplification reaction, the DNA capture beads were recovered and enriched for beads carrying DNA fragments (GS FLX Titanium SV emPCR kit and GS FLX Titanium emPCR Breaking filters, SV; Roche). For the sequence reaction the GS FLX Titanium Sequencing kit XL700 (Roche) was used in combination with the matching GS FLX Titanium PicoTiterPlate Kit 70 × 75 (Roche) with each sample on 1/56 of the plate. The Sequencing run was performed at the Academic Medical Center of the University of Amsterdam on the Genome Sequencer FLX instrument (454 Life Sciences; Roche).

The raw sequence data, assembled per sample (Exp1 and Exp2), were loaded in Codon Code Aligner 3.5.6. and the A- and B-adapters were trimmed. All similar or overlapping reads were assembled in contigs. Since one restriction enzyme was used partial overlapping fragments are rare. After assembling, the contigs and unassembled sequences were exported as FASTA files. The FASTA files were uploaded in NCBI BLAST nucleotide (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with the GenBank Nucleotide collection nr/nt database. The output was saved as text files and imported to MEGAN Analyzer (version 3.8, 4 February 2010), a program that assigns the reads to the different taxa of the NCBI taxonomy using a straightforward assignment algorithm (Huson et al., 2007). Viral sequences were registered. Codon Code Aligner 3.5.6. was used to align the VIDISCA–454 fragments to known viral sequences.

In order to differentiate between host and experiment specific sequences, FASTA files were compared locally with vertebrate mammalian RNA and microbial RNA (both NCBI) by the Blast tool. A minimum homology of 90% and query coverage of 75% were used as the threshold. The remaining contigs were aligned with BLASTn against viral genomic sequences to detect known viral sequences. A homology of 90% and query coverage of at least 35% were required

### Table 1

<table>
<thead>
<tr>
<th>Adapter</th>
<th>Sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Adapter</td>
<td>GCTTGGAGCCCCAGCACAGA</td>
</tr>
<tr>
<td>Bottom</td>
<td>TATTCGACCGGGTTGAAAA GGCC</td>
</tr>
<tr>
<td>A-Adapter MID7</td>
<td>GCCCTCTCTCGCATAGCGTCCTCA</td>
</tr>
<tr>
<td>Bottom</td>
<td>TATTAGACACCATGCATGCGCAGCC</td>
</tr>
<tr>
<td>A-Adapter MID8</td>
<td>GCCCTCTCTCGCATAGCGTCCTCA</td>
</tr>
<tr>
<td>Bottom</td>
<td>TATGACACCGGACCTGATGCGCAGCC</td>
</tr>
</tbody>
</table>

---

**References**

1. Ambion, Nieuwekerk a/d IJssel, The Netherlands.
2. A- and B-adapters (Roche).
3. A- and B-adapters were trimmed. All similar or overlapping reads were assembled in contigs.
4. A homology of 90% and query coverage of at least 35% were required.
to assign a fragment to the particular virus. The number of unique viral fragments was registered.

Three days after the first inoculation of the canine BDE cell line, cell death was observed and cells with a variable morphology were present. After the second inoculation considerable cell damage, rounding of cells and cell death was observed. At the end of the cell culture experiments clear cytopathogenic effect was seen with extreme cell death, grape-like clusters, and many detached cells. The results of the duplicate experiment (Exp2) were consistent with the results of the first experiment (Exp1).

The cell culture supernatant of both cell culture experiments was examined by VIDISCA-454. In Exp1 4951 fragments, with a read length varying from less than 20 nucleotides to a maximum of 737 nucleotides, were sequenced. After trimming of the adapters, 4857 sequences (length > 20 nucleotides) remained. Manual analysis of the fragments showed immediately that the majority of the sequences were similar to Canine adenovirus type 1. Therefore this virus was used as the reference sequence (RefSeq Acc. ID: AC_000003.1) in order to create an alignment of the fragments in the Codon Code Aligner application. This alignment revealed that the fragments were able to cover almost the complete genome of CAV-1. In total 4137 CAV-1 fragments were found in Exp1, corresponding to a viral sequence yield of 85 percent. To assess the number of unique CAV-1 fragments in Exp1, identical fragments were assembled in contigs. As a result 228 unique CAV-1 sequences were registered. Exp2 revealed similar results and contained 4334 sequences (length > 20 nucleotides) after trimming of the VIDISCA-454 adapters. Alignment of the Exp2-fragments against the CAV-1 reference sequence (RefSeq Acc. ID: AC_000003.1) also showed nearly full genome coverage of the CAV-1 virus. Furthermore, a viral sequence yield of 83% (3601 of the 4334 fragments were similar to CAV-1) is comparable to the viral sequence yield of Exp1. Finally, 231 different CAV-1 fragments were identified in Exp2, which equals the amount of unique CAV-1 fragments found in Exp1. After merging all the fragments of Exp1 and Exp2, a viral sequence yield of 84% (7738 of the 9190 fragments were considered to be CAV-1) was obtained. In conclusion both cell culture samples contain CAV-1.

The VIDISCA-454 technique demonstrated clearly that the dog had suffered from a CAV-1 infection. It was possible to assign 84% of the DNA fragments to CAV-1 and almost the full genome of this virus was recovered, even though CAV-1 infection was not fully expected based on the histopathological features (absence of viral nuclear inclusions) of this dog. Furthermore, this four-year-old dog acquired the CAV-1 infection at a relatively old age, whereas hepatitis contagiosa canis occurs mainly in more susceptible younger dogs and is characterized by severe necrosis and many viral nuclear inclusions (Decaro et al., 2008). Due to vaccination strategies hepatitis caused by CAV-1 had disappeared almost completely, however, in Europe, CAV-1 is re-emerging and outbreaks of the disease have been reported in unvaccinated dogs and foxes (Thompson et al., 2010; Decaro et al., 2007). The dog in this study did not receive all the recommended vaccinations and remained therefore susceptible for CAV-1 infection. Gocke et al. (1967) infected partially immune dogs with CAV-1, which lead to the development of chronic hepatitis in seven of the eleven experimental dogs. The other four dogs developed a sub-acute form of the disease, instead of the usual acute fulminating hepatitis observed in fully susceptible dogs. Nonetheless, involvement of CAV-1 infection in canine idiopathic primary hepatitis could not be demonstrated by molecular or immunohistochemical techniques (Boomkens et al., 2005).

In the present study, canine liver tissue material obtained from a dog deceased as a result of long-lasting hepatitis was cultured on a canine Bile Duct Epithelial (BDE) cell line. The availability of a dog cell line of hepatic origin permitted us to enrich the viral load of the samples analyzed, and to reduce background sequence information of the host. Although CAV-1 viruses are cultured generally on Madin Darby Canine Kidney cells (Thompson et al., 2010; Decaro et al., 2008), this study shows that canine BDE cells are also suitable for viral amplification. After three inoculations, a distinctive cytopathogenic effect, including grape-like cell clusters typical of CAV-1 (Decaro et al., 2008), was observed. In spite of the high level of arginase present in liver tissue, which inhibits CAV-1 growth in cell cultures (Decaro et al., 2008; Carmichael, 1972), the adenovirus was able to replicate in the BDE cell line. This could be due to a considerable viral load in the liver tissue samples, or following a wash-out of arginase in the course of the cell culture experiment. In conclusion, because BDE cells are an established model for dog hepatocytes (Spee et al., 2008) and, in addition, are capable of promoting adenovirus replication, this cell line can be a valuable in vitro model for the study of CAV-1 infection in dogs and in CAV-1 vaccination studies.

This is the first report on the use of the VIDISCA-454 technique for detection of a viral etiolog in canine idiopathic hepatitis. Although the technique proves clearly its ability to detect a virus in canine samples without any prior knowledge about the viral species involved, it will remain a Herculean task to deduce new viruses from sequence reads that do not align to known viruses. The problem with the detection of new viruses is the lack of a database for the comparison of the obtained sequences, making the data analysis complicated and time consuming. Notwithstanding these limitations, unknown viruses from animals have been discovered recently (e.g. Day et al., 2010; Li et al., 2010), including a new canine hepacivirus (CHV) that currently seems more linked to respiratory infections in dogs than to canine hepatitis (Kapoor et al., 2011).

The application of VIDISCA and next generation sequencing in canine hepatitis research now permits the detection of unknown viruses, not based on any previous knowledge of sequence homology with known viruses. Such a new approach should lead to the discovery of other viruses associated with the majority of canine hepatitis cases in the veterinary clinic.

Acknowledgements

This study was supported by funding from VIDI grant 016.066.318 from The Netherlands Organization for Scientific Research (NWO), European Community's Sixth Framework Programme under the project GRACE, EC grant agreement number LSHM-CT-2005-518226, and European Community's Seventh Framework Programme (FP7/2007 – 2013) under the project EMPERIE, EC grant agreement number 223498.

References


