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# The application of single strand conformation polymorphism (SSCP) analysis in determining *Hepatitis E virus* intra-host diversity

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Genetic heterogeneity of RNA populations influences virus pathogenesis, epidemiology and evolution. Therefore, accurate information regarding virus genetic structure is highly important for both diagnostic and scientific purposes. For the *Hepatitis E virus* (HEV), the causal agent of hepatitis in humans, the intra-host population structure has been poorly investigated, mainly using the less sensitive RFLP-based approach. The objective of this study was to assess the suitability and the accuracy of single strand conformation polymorphism (SSCP) analysis, a well-established tool in genetic variation research, for the characterization of HEV quasispecies. The analysis was conducted on 50 clones of five swine isolates and 30 clones of three human HEV isolates. To identify and quantify the sequence variants present in each HEV isolate, 348 bp long fragments of the amplified conserved ORF2 region were separated by cloning. Ten clones per isolate were subjected to SSCP and sequenced in a parallel experiment. The results show a high correlation of SSCP haplotype profiling with the sequencing results, confirming the sensitivity and reliability of this simple, rapid and low cost approach in the characterization of HEV quasispecies.



## 1. Introduction

Intra-host RNA viral populations are composed of genetically related variants (Ojosnegros et al., 2011; Domingo et al., 2012) due to their high mutation and replication rates. These quasispecies are dynamic sources of viral adaptability enabling rapid evolution during different selective regimens (Schneider and Roossinck, 2001; Domingo et al., 2006). Evidently, virus pathogenicity depends on the structure of the quasispecies and the abundance of certain virus variants (Kumar et al., 2008; Ojosnegros et al., 2011). Quasispecies can hide components that in isolation would display dissimilar biological properties (Černi et al., 2008; Domingo et al., 2012), while reinfections may play a role in the accumulation of highly heterogeneous virus variants, some of which are distant phylogenetically. Hence, it is important to have an efficient laboratory screening tool for quick and accurate characterization of virus isolates.

Although bulk cloning and sequencing is a good approach for detecting viral heterogeneity, it requires the preparation and sequencing of large numbers of clones to ensure that minor sequence variants are represented. Mainly due to the cost of too many man-hours, it is not the most cost effective approach. Furthermore, the next generation sequencing approach, which enables quick detection of single nucleotide polymorphisms within different haplotypes, is still challenged with the reconstruction of virus quasispecies (Schirmer et al., 2012) and is not available widely for routine screenings. Single strand conformation polymorphism (SSCP) analysis is a good alternative that provides excellent insight into viral heterogeneity and reduces research costs (Gasser et al., 2006). This method enables the electrophoretic separation of single strand nucleic acids where a single nucleotide change could considerably affect strand electrophoretic mobility by altering intra-strand base paring and its resulting conformation (Orita et al., 1989). To date, it has been used widely for the rapid screening of selected genome fragments to identify most sequence variations between the mutants of many viruses, including hepatitis A, B and C (Hardie et al., 1996; Mackiewicz et al., 2005; Kumar et al., 2008).

Although *Hepatitis E virus* (HEV) has only one serotype (Emerson and Purcell, 2003), its isolates display considerable genetic diversity (Lu et al., 2006; Smith et al., 2013). Increased testing and sequencing availability have resulted in an increased number of recorded HEV variants from wildlife and the environment (Smith et al., 2013). The recent *Hepeviridae* taxonomy proposal (Smith et al., 2014) recognizes four HEV human-infecting genotypes within the species *Orthohepevirus* A, though clear criteria are yet to be defined for the delineation of different HEV subgenotypes. To date, HEV

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genotypes 1 and 2 have been found to infect only humans. However, the zoonotic genotypes 3 and 4 have been detected in humans and in several animal species (Meng, 2010; Teshale et al., 2010). Domestic pigs are considered to be the main HEV reservoirs and have been studied the most extensively (Meng, 2011; Van der Poel, 2014).

The quasispecies nature of HEV was first described by Grandadam et al. (2004) using restriction fragment length polymorphism (RFLP) analysis. Though two studies to date have addressed the direct sequencing of 20 randomly selected HEV clones (Zhang et al., 2010; Lhomme et al., 2012), RFLP remained the only method used in the characterization of intra-host heterogeneity of HEV.

In this study, the suitability of SSCP, a method for the simultaneous detection of DNA polymorphisms at multiple positions (Orita et al., 1989; Gasser et al., 2006), was tested for the detection of HEV variants originating from swine and human samples. Clones containing a widely used conserved fragment of the HEV capsid protein (CP) gene (Meng et al., 1997; Huang et al., 2002; Lu et al., 2006), which is important phylogenetically and diagnostically, were subjected to SSCP analysis and sequencing under the assumption of sufficient SSCP reliability and sensitivity to characterize HEV intra-host populations.

## 2. Materials and methods

### 2.1. HEV isolates

Five swine (CRO_2D, CRO_5D, CRO_3F, CRO_22F and CRO_5W) and three human serum samples (8, 16 and 22), all previously confirmed to be HEV positive, were analysed. Swine samples originating from four different Croatian counties were collected through the Classical Swine Fever National Monitoring Programme, in line with the standard international protocols for animal welfare. The samples CRO_2D and CRO_5D originated from fattening pigs bred on two small farms; CRO_3F and CRO_22F originated from sows bred on a large industrial farm; while sample CRO_5W originated from a wild boar. All five samples tested HEV positive by ELISA (MP Diagnostics HEV ELISA kit, Medical Technology Promedt, St. Ingbert, Germany). Human samples used in this analysis were deposited by Dr. A. R. Ciccaglione in the serum collection at the Istituto Superiore di Sanità (Rome, Italy) and purified 457 bp long PCR products of ORF2 region were kindly provided by Dr. G. La Rosa. Samples 8 and 22 originated from acutely infected Italian patients, while sample 16 was obtained from an acutely infected patient from Bangladesh. All human samples were previously confirmed to be HEV positive, both serologically and molecularly, as described by La Rosa et al. (2014).

### 2.2. cDNA synthesis and PCR amplification

Swine sera were separated from cellular elements by centrifuging coagulated blood (blood clots were rimmed with a sterile glass stick to facilitate separation) for 15 min at 1000 g. For each serum sample, a 140 μl aliquot was used for viral RNA purification using the QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA samples were stored at −80 °C until analysis.

RNA samples were reverse transcribed by SuperScript III reverse transcriptase using random hexamers (Invitrogen, Life Technologies) following the manufacturer's instructions. The generated cDNA was used immediately for polymerase chain reaction (PCR) or stored at -20 °C. Two sets of HEV primers (ORF2-3156/ORF2-3157 - direct and ORF2-3158/ORF2-3159 - nested) amplifying a 348 bp long fragment of the ORF2 conserved region (Huang et al., 2002) were used for the universal HEV detection by nested PCR assay. The direct and nested step PCR parameters were identical (initial denaturation: 94 °C, 3 min; 30 cycles: denaturation: 94 °C, 1 min; annealing: 50 °C, 1 min; extension: 72 °C, 1 min; final extension: 72 °C, 20 min).

For human samples, the provided 457 bp PCR products were subjected to the nested PCR using a primer pair (ORF2-3158/ORF2-3159) and PCR parameters as described for swine samples. All PCR products were subjected to electrophoresis in 1.5% agarose gel and stained with ethidium bromide for UV light visualization.

### 2.3. Separation and identification of HEV variants

To separate different genomic variants, nested PCR amplicons of each isolate were TA-cloned into the pTZ57R/T vector (Fermentas, Vilnius, Lithuania) as per manufacturer recommendations. Competent *Escherichia coli* XL-1Blue cells and their transformation were prepared using a commercial InsTAclone PCR cloning kit (Fermentas, Vilnius, Lithuania). Transformed colonies were selected by α complementation, and the presence of the insert was confirmed by PCR using the same primer pair and nested PCR reaction conditions as described above. Ten transformed colonies per isolate were selected randomly and subjected to SSCP.

Aliquots of the amplified products (1 μl) were added to 9 μl denaturing solution (95% formamide, 20 mM EDTA, pH 8.0, and 0.05% bromphenol blue), heated for 5 min at 90 °C and immediately put on ice. Denatured products were separated by electrophoresis in native 8% polyacrylamide 0.75 mm thick gel in the standard TBE buffer under constant voltage (200 V) at 4 °C for 3 h in the Mini PROTEAN Tetra Cell (Bio-Rad Laboratories, Hercules, CA, USA). The SSCP profiles were visualized by silver staining (Beidler et al., 1982) and PCR products displaying different SSCP patterns were considered different genomic variants (López-Labrador et al., 1999; Kong et al., 2000). Plasmids from all ten transformed colonies per samples (80 clones in total) were purified using a PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, Lőhne, Germany) and sequenced in both directions (Macrogen, Seoul, Korea) using a pair of M13-pUC universal primers.

### 2.4. Nucleotide sequence and population structure analysis

To determine the phylogenetic clustering of the obtained sequences, the reference ORF2 sequences and representatives of all *Orthohepevirus* A HEV genotypes (Smith et al., 2014) were retrieved from GenBank. Sequences were aligned using ClustalX 1.8 (Thompson et al., 1997) and analysed using MEGA 5 (Tamura et al., 2011). The phylogenetic tree was generated using the maximum likelihood method applying the Tamura Nei evolutionary model, selected as the most appropriate after performing Modeltest 3.7 analysis (Posada and Crandall, 1998). Tree topology was evaluated by bootstrap analysis based on 1000 repetitions. The overall mean genetic distance was calculated using MEGA 5 (Tamura et al., 2011) for the quasispecies of each sample. Population structure was considered at the haplotype level as determined by the SSCP analysis and compared to the structure obtained at the nucleotide sequence level. All sequences displaying nucleotide differences obtained in this work were submitted to GenBank under accession numbers KF366506-KF366524 and KP878281-KP878299.

## 3. Results

The nested RT-PCR assay, using primers specific for HEV ORF2, gave strong amplification signals corresponding to the expected 348 bp long products in all tested samples. The comparison of SSCP patterns obtained after the analysis of 10 randomly selected genomic variants of each sample, separated by TA-cloning, suggested the coexistence of different genomic variants within all



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