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OBSERVATIONS ON THE QUASISPECIES COMPOSITION OF THREE ANIMAL PATHOGENIC RNA VIRUSES

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The quasispecies nature of three animal pathogenic RNA viruses of field origin was examined by testing variants of classical swine fever virus (CSFV) originating from geographically different areas, feline coronavirus (FCoV) detected from the same animal by successive sampling, and rabbit haemorrhagic disease virus (RHDV) originating from successive outbreaks in the same geographic area. Clinical samples were investigated using reverse transcriptase polymerase chain reaction (RT-PCR) and ensuing single strand conformational polymorphism (SSCP) assay. By the combination of these methods even subtle differences could be detected among the amplified fragments of the same virus species of different origin. FCoV proved to comprise the most and CSFV the less heterogeneous virus quasispecies. The results show that the combination of RT-PCR and SSCP provides novel and highly sensitive means for the characterisation of RNA viruses, with special regard to genome composition, evolution, features of pathogenicity and molecular epizootiology.

Key words: CSFV, FCoV, RHDV, RT-PCR, SSCP, quasispecies

The formation of '*quasispecies*' population during replication has been observed for several RNA viruses (Holland et al., 1992; Studdert, 1994; Gunn-Moore et al., 1998; Radford et al., 1998). The term refers to diverse, rapidly evolving and competing populations of the RNA genome. The sequence of the most fitting genome, which gives the vast majority of the replicating variants, is termed 'master sequence', while the competing virus variants are named the 'mutant spectrum'. The importance of the quasispecies nature of RNA viruses lies in their ability to evolve rapidly due to the extremely high mutation frequencies per site in their genomes (Holland et al., 1992). The concept of this phenomenon is not new, because very high mutation frequencies for phenotypes such as temperature sensitivity, pathogenicity or plaque size mutants were already reported decades ago (Granoff, 1961; Eggers and Tamm, 1965). However,

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the newly developed techniques provide novel and effective means to study the quasispecies populations. The recent studies revealed that a given quasispecies variant gradually changes even in a constant environment with time, but the fastest ways for these events are associated with the changes of the environment, i.e., during development of immune responses, changes of host species, targeting a new cell type by the virus within a single host, interference by defective viruses, inflammatory responses, etc. (Kurosaki et al., 1994). Consequences of changes of RNA virus quasispecies populations comprise rapid antigenic change and immune system evasion, as well as rapid development of effective resistance to antiviral drugs (Holland et al., 1992). Such diversity also facilitates the establishment and progression of chronic infections. A heterogeneous swarm of RNA genomes in the infected tissues then renders it impossible to assign specific pathological significance to any consensus sequence recovered from the diseased tissue, hence making it difficult to fully explore the pathogenesis of infections (Holland et al., 1992).

Despite the general knowledge that RNA viruses are quite mutable and adaptable, their extreme mutation frequencies have been documented only recently with the advent of rapid techniques for genome mapping (Holland et al., 1992). The most widely applied method exploits the power of the PCR that can detect and amplify the targeted genome region even from a trace amount of the examined pathogen. The generated PCR products are then investigated further in the high-resolution single strand conformational polymorphism (SSCP) assay (Kurosaki et al., 1994). In the SSCP assay the single stranded PCR products, generated in asymmetric PCR, are loaded onto concentrated non-denaturing polyacrylamide gels where the fragments run in secondary conformation determined by their nucleotide sequences. The resolution of the technique can be as high as 99% ability to detect a single nucleotide change when running fragments shorter than 350 nucleotides (Hayashi, 1991). With the increase of the length of the fragments the resolution gradually decreases. Depending on the sensitivity of the detection (staining) method the number of the revealed subpopulations will differ. There are various methods to increase the sensitivity or to obtain more information about the subpopulations. For example, the selected subpopulations can be re-amplified and sequenced to gain the ultimate information about their nucleotide composition (Lázaro and Estivill, 1992; Martell et al., 1992).

In this report we demonstrate that the composition of the quasispecies of the same virus species varies when comparing strains that differ either in geographic origin or in time and, additionally, which occur in the same animal during chronic infection. The selected agents are classical swine fever virus (CSFV) representing the Flaviviridae family, rabbit haemorrhagic disease virus (RHDV), a member of the Caliciviridae family, and feline coronavirus (FCoV) from the Coronaviridae family.

Materials and methods

Viruses

The examined viruses comprised field isolates collected at the Veterinary Institute, Debrecen, Hungary. CSFV was obtained from archive spleen samples of necropsied animals affected by the disease (Kiss et al., 1999*a*). The samples originated from six different areas of Hungary from outbreaks during the late 1980s. FCoV originated from the rectal swab of a clinically healthy, virus-carrier cat which was monitored on eight occasions with ten-day intervals. RHDV was obtained from the livers of animals succumbed to the disease. The samples represented outbreaks that took place in four different years in the same geographic region of Hungary.

Sample preparation and RNA extraction

In the case of CSFV tissue samples of the spleen were treated as described elsewhere (Kiss et al., 1999*a*). The RHDV-infected samples were homogenised in sterile distilled water (10%, w/v), while the FCoV swabs were vortexed in 1.5 ml of bidistilled water, then the specimens were centrifuged at 5,000 × *g* for 5 min to pellet the insoluble components. The supernatants were stored at –70 °C or used immediately depending on the actual number of samples.

The RNA preparation was carried out after Boom et al. (1990) and Cheung et al. (1994), and was as follows: 100 µl of the suspensions was added to 20 µl of size-fractionated silica in 900 µl of lysis buffer (120 g guanidine thiocyanate dissolved in 100 ml of 0.1 M Tris-HCl [pH 6.4], 22 ml of 0.2 M EDTA [pH 8.0], and 2.6 g of Triton X-100). The samples were vortexed and left for 10 min at room temperature. The supernatant was removed after a quick spin (12,000 × *g* for 1 min), and the silica-RNA pellet was washed twice with 450 µl of washing buffer (120 g of guanidine thiocyanate dissolved in 100 ml of 0.1 M Tris-HCl [pH 6.4]) and twice with 1 ml of 70% (v/v) ethanol. After a final wash with 1 ml of acetone, the pellet was dried at 56 °C for 10 min. The RNA was eluted by resuspending the pellet in 40 µl of diethyl pyrocarbonate (DEPC)-treated water at 56 °C for 10 min. The supernatant recovered after pelleting the silica particles (12,000 × *g* for 3 min) contained the RNA and was used for reverse transcription.

Synthesis of cDNA

CSFV cDNA was synthesized by using the First Strand cDNA Synthesis kit (Pharmacia, Uppsala, Sweden) as recommended by the manufacturer, with the exception that half amount of the random hexamers was replaced by the reverse PCR primer (see Table 1; Kiss et al., 1999*a*). RHDV cDNA was synthe-

sised in 28 µl reaction mixtures as described earlier (Ros Bascuňana et al., 1997), and FCoV RNA was treated in the same way. A preliminary mixture containing 5 µl of RNA, 5 µl of DEPC-treated water, and 1 µl of random hexamers (0.02 U; Pharmacia) was incubated at 65 °C for 5 min for RNA denaturation. Subsequently, the tube was placed on ice, and 17 µl of a premixture containing 2.5 µl of each deoxynucleotide triphosphate (2 mM; Pharmacia), 5 µl of 5 × reaction buffer (0.25 M Tris-HCl [pH 8.3], 0.375 M KCl, 15 mM $MgCl_2$), 1 µl of RNAguard (24 U; Pharmacia) and 1 µl of Moloney murine leukaemia virus reverse transcriptase (200 U; Gibco BRL, Bethesda, MD, USA) was added. The reaction mixture was incubated at 37 °C for 90 min, followed by incubation at 98 °C for 5 min to inactivate the enzyme.

Primers

The design of the primers used in the experiments has been described elsewhere (Ros Bascuňana et al., 1997; Kiss et al., 1999*a*), and is shown in Table 1.

Table 1

Primers used for the amplification of CSFV, RHDV and FCoV

Virus	Primers	Sequence (5' to 3')	Position
CSFV	PEST3 (F)	ATATATGCTCAAGGGCGAGT	3378-3398[a]
	PEST6 (R)	ACAGCAGTAGTATCCATTTCTTTA	3664-3686[a]
RHDV	REF (F)	CAACCTCCAGCCCACCACCAACAC	6462-6485[b]
	REB (R)	TGGTTGGGAGCCTGTGCCGTACTG	6754-6777[b]
FCoV	OFIP1A (F)	ATTTTGGAATTTATGTCCGAGAGA	990[c]
	OFIP2A (R)	CTAGCACCATAGAAAGTTGTCACA	1574[c]
	OFIP3 (F)	CGCTGAGAGGTGGTTCTTTTACTTC	1110[c]
	OFIP4 (R)	CTTCCAGGTGTGTTTGTTGGCATTC	1554[c]

[a]Katz et al. (1993) E2 (gp53) region; [b]Meyers et al. (1991) VP60 gene; [c]Vennema et al. (1991) nucleocapsid protein coding region. F, forward primer, R, reverse primer. The expected lengths of the products are as follows: CSFV, 308 bp; RHDV, 316 bp; and FCoV (with primers OFIP3-OFIP4), 444 bp

PCR procedure

The PCR assays were carried out in 50 µl volumes. Each reaction mixture contained 5 µl of 10 × GeneAmp PCR buffer II (Perkin-Elmer Cetus, Norwalk, CT, USA), 15 pmol of each primer (in the case of FCoVs OFIP1A and OFIP2A for the first reaction), 5 µl of bovine serum albumin (1 mg/ml), 1 µl of each deoxynucleotide triphosphate (10 mM; Pharmacia), 2.5 mM $MgCl_2$, 1 U AmpliTaq DNA polymerase (Perkin-Elmer Cetus) and 2 µl cDNA. Two droplets of mineral oil (Sigma) were added to prevent evaporation. In the case of FCoV nested PCR

was carried out to achieve the desired sensitivity by using the OFIP3 and OFIP4 primers and 2 µl reaction mixture of the first PCR as target. The thermal profiles are shown in Table 2. By using the primers in 1:10 ratio in order to produce excess amount of single stranded PCR products, 1 µl amounts of the completed PCR mixtures were subjected to asymmetric PCR.

Detection of the PCR products

The amplified DNA products were analysed by electrophoresis on 2% agarose gels (SeaKem ME, FMC Bioproducts, Rockland, Maine, USA) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) containing 0.5 µg/ml ethidium bromide. Gel electrophoresis was performed at a constant voltage of 110 V for 45 min, and the size of the products was estimated using a 100 bp DNA ladder.

Table 2

The cycling parameters used for the PCR assays

	Denaturation	Primer annealing	Primer extension*	No. of cycles
CSFV	94 °C for 30 s	55 °C for 30 s	72 °C for 30 s	35
RHDV	94 °C for 30 s	60 °C for 30 s	72 °C for 30 s	35
FCoV I	94 °C for 45 s	50 °C for 60 s	72 °C for 2 min	5
	94 °C for 45 s	45 °C for 60 s	72 °C for 2 min	30
FCoV II	94 °C for 45 s	52 °C for 1.5 min	72 °C for 3 min	30

*The final extension step was 72 °C for 7 min in each procedure. FCoV I indicates the cycles with OFIP1A and OFIP2A primers, FCoV II means the cycles with the nested primers (OFIP3 and OFIP4).

SSCP analysis

The asymmetric PCR reaction mixtures in 5 µl amounts were added to 15 µl SSCP loading buffer (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue), kept at 95 °C for 5 min, put on ice for 5 min, then loaded onto 10% and 7.5% polyacrylamide gels for the 440 and approx. 310 nucleotides long fragments, respectively, to gain the highest possible resolution. The gels were run in a Mini Protean Cell (Bio-Rad, Hercules, CA, USA) at 140 V constant power for 2–4 h at room temperature. Subsequently, the gels were submerged into a 0.5 µg/ml ethidium bromide solution for 5 min, then visualised under ultraviolet light and photographed.

Results and discussion

Figures 1a, b, and c show the results of the PCR assays. The specific bands are seen at the expected sizes. Figures 2a, b, and c show the results of the SSCP analysis. The consistence of the patterns was confirmed by repeatedly accomplished PCR-SSCP analyses (data not shown). The distinct bands in the same lane represent subpopulations with different nucleotide sequences (Lázaro and Estivill, 1992). The slight variations of the master sequences are clear and, interestingly, the greatest difference is seen between the patterns of two RHDV samples originating from the same outbreak. The variation in the swarm of the mutant spectrum is exceptionally represented by FCoV, where continuous changes of the number of the escorting subpopulations (from two to seven) with a stable master sequence can be observed in the same individual sampled successively. As can be followed, new subpopulations may arise, then appear intermittently within short periods of time (10 days in our case). Such phenomena were observed in patients chronically infected with hepatitis C viruses (Martell et al., 1992; Kurosaki et al., 1994), and it was emphasised that these changes were not merely the result of the pressure of the immune system but, rather, they occurred spontaneously (Domingo et al., 1993). Apparently, the CSFVs proved to contain very few if any sequences in the mutant spectrum compared to the other two viruses, though the envelope glycoprotein E2 that was targeted by the RT-PCR is the most variable protein of pestiviruses (van Rijn et al., 1997). Our finding is in harmony with the nucleotide sequence data, since it was established that CSFVs in the field show relative genetic stability, thus even small sequence differences should have epizootiological significance (Stadejek et al., 1997; Vilček and Paton, 1998).

A remarkable point is that for tracking the evolution of the quasispecies it is better to target the structural protein coding regions of the virus genome, since the non-structural protein coding parts show much less variation. This finding is noteworthy from the aspect of pathogenesis as well (Kiss et al., 1999*b*). Thus, for the thorough monitoring of the changes of a virus quasispecies it is inevitable to analyse several parts of the virus genome.

The assumption that a particular RNA virus and, thus, a particular RNA virus disease does not exist was suggested earlier (Holland et al., 1992), though the concept of quasispecies must be approached with care (Smith et al., 1997). Nevertheless, it has already been demonstrated that the prevalent subpopulations of feline coronaviruses differ between healthy and clinically affected cats (Gunn-Moore et al., 1998). Marked changes were observed in virus neutralisation profiles of feline caliciviruses due to the evolving quasispecies in persistently infected cats (Radford et al., 1998).

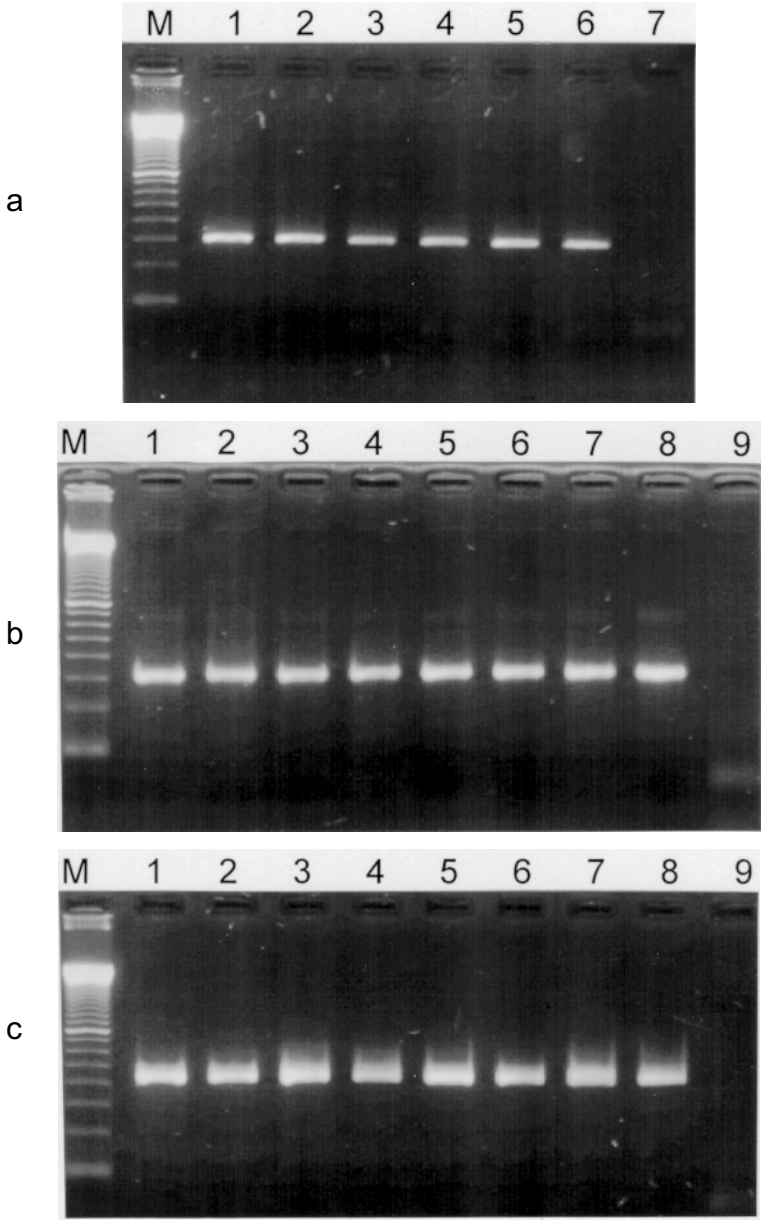

Fig. 1. Results of the PCR assays performed on the different samples of CSFV, RHDV and FCoV. Each lane M contains a 100 bp molecular weight marker. In Fig. 1a lanes 1–6 represent different settlements where an outbreak of classical swine fever was recorded at approximately the same time; lane 7: negative control. In Fig. 1b the four consecutive outbreaks caused by RHDV are represented by two samples (lanes 1–2, 3–4, 5–6 and 7–8) each. Lane 9: negative control. Fig. 1c shows the RT-PCR results of FCoV collected on eight successive occasions with ten-day intervals (lanes 1–8); lane 9: negative control

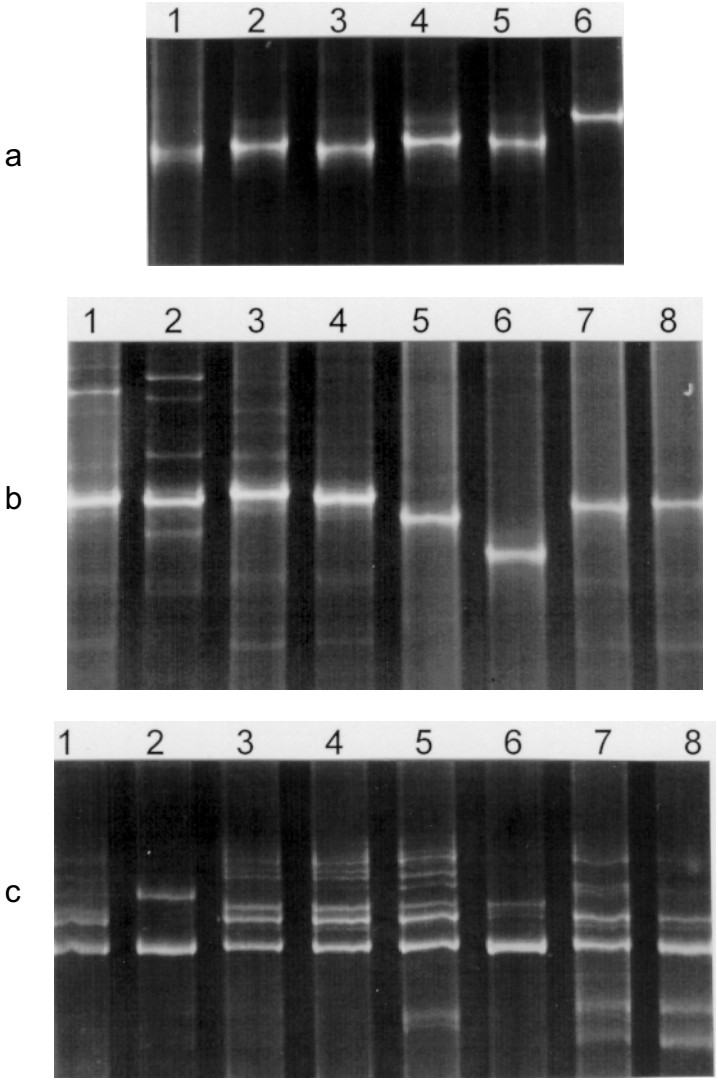

Fig. 2. SSCP patterns of the PCR products shown in Fig. 1 after re-amplification in asymmetric PCR. Fig. 2a, 2b and 2c shows the patterns of CSFV, RHDV and FCoV, respectively

Data obtained from this study and from further similar investigations may be useful in pathogenicity studies and in vaccine development as well: a reasonably stable viral genome (i.e., with few subpopulations) indicates that even slight variations in nucleotide composition may present significant biological consequences compared to highly variable virus genomes (with a large number of virus subpopulations) that rather establish chronic infections, and changes in their nucleotide composition rarely produce serious disorders.

Of course, the ultimate and most accurate characterisation is obtained by nucleotide sequencing. However, by inserting the SSCP between the PCR and nucleotide sequencing, at least two advantages are apparent: (*i*) unnecessary sequencing steps can be avoided in the case of the same SSCP patterns; (*ii*) when it is reasoned, the desired band (representing one subpopulation) can be crushed from the SSCP gel and re-amplified, then sequenced to characterise a particular subpopulation (Kurosaki et al., 1994).

Based on its simplicity, rapidity, and its informative force the presented combination of the basic molecular techniques will hopefully be applied for studies targeting the pathogenesis of viral diseases, and is also recommended for diagnostic purposes, though primarily for particular rather than for routine investigations.

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