Genetical characteristic of six strains of RHD (rabbit haemorrhagic disease) virus originating from Europe

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Abstract

Rabbit haemorrhagic disease virus (RHDV) is a noncultivable calicivirus that infect rabbits (Oryctolagus cuniculus) and cause epidemics in population of those animals across whole the world. The phylogenetic analysis of different strains of RHDV originated from different countries showed that homology of nucleotide sequences is 92-100%. The aim of the study was genetic characteristic of six Central European strains of RHD virus (Eisenhuttenstadt, Frankfurt, Rossi, V411, 24/89, 1447/96) on the basis on molecular analysis of a 510 bp fragment, coding N-terminal and 320bp fragment coding C-terminal part of structural VP60. The analysis was conducted to lead to determine the phylogenetic dependence of chosen RHD strains. The analysis of six European strains of RHD virus showed that the changeability of those strains is low, ranging from 1 to 11%.

Key words: *RHD virus, polyprotein VP60, phylogenetic analysis, genogroup.*

(Centr Eur J Immunol 2009; 34 (1): 7-13)

Introduction

The RHD virus is an etiological agent responsible for rabbit plague (RHD – rabbit haemorrhagic disease), which was classified into the *Caliciviridae* family in 1992. The virus was first described in 1984 in China [quoted by 1, 2], while in Europe first records of the disease come from 1986 [quoted by 1, 2]. Presently, the virus is present in Asia, Europe, Central and South America, Africa, Australia and New Zealand, and recently also in the USA, causing great damage to breeding of the animals [quoted by 1,2].

RHDV is a small envelope-free virus with genetic material in the form of one-strain, linear, positively polarised RNA composed of 7437 nucleotides [3, 4]. Genetic analysis showed that the genome contains genes encoding non-structural proteins including helicase, protease, and RNA-dependent polymerase, and structural VP60 capsid protein with molecular weight of 60 kDa [5-7]. It was also shown that the amino acid sequence of VP60 protein is divided into 6 regions (A-F), similarly as in other caliciviruses [8], whereas C and E regions and hyper-variable regions. Capsid of the RHD virus comprises two domains: the internal one is formed of the N-terminal (amino) fragment of VP60 protein, which at the amino end contains S domain that protects the genome, while the external domain is created by the C-terminal (carboxy) fragment of VP60, containing P2 domain, where two hyper-variable C and E regions are located, among six found in VP60 [9-12].

Presently, in 32 strains of the RHD virus received in the years 1987-2006, full nucleotide sequence was recognised [13]. Also, in 35 strains, full sequence is known of the gene (1740 nucleotides) encoding structural VP60 protein, and in over 200 strains, fragmentary sequences have been described, principally of the gene encoding VP60, and genes encoding other proteins [13]. These study was aimed at showing similarities and differences between such strains and learning about the ways of the virus spreading across the world, although it was also help to indicate the elements of the genome that could be used to construct a vaccine against rabbit plague [14, 15].

A comparative study of full nucleotide sequences of the RHD virus genome recorded 96% of similarity between the

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German FRG strain and the French SD strain [16]. Also large similarity, as much as 99%, was recorded between the FRG strain and the Czech V-351 strain, and 95% of similarity between V-351 and the Spanish AST89 [17, 18]. The study of 44 English strains of RHD virus, based on the sequence of the gene encoding VP60, showed their homology at the level of 92% between British strains and just 86% of similarity as compared to 17 European strains [19]. Similar results were obtained in the study of Nowotny et al. [15], analysing 44 European strains based on fragment of the gene encoding VP60, and by Fitzner et al. [1, 20, 21], who studied 8 Polish strains on the basis of the fragment of the gene encoding VP60 and the fragment of the gene encoding non-structural protein. The authors shown that variability between the strains ranged from 1-2.8% [1, 20, 21] to 14% [15]. High homology (93-96%) was recorded in the study of 4 Czech strains [2], where the analysis referred to the fragment of the gene encoding VP60. Also, the studies of Capucci's team [9] regarding 2 Italian strains identified in 1997 revealed homology at the level of 98.5%. Rather high levels of homology, ranging from 90.5% to 100%, was also recorded among 17 Hungarian strains collected in the years 1988-2003 [22], 4 French strains from 2001 [23], 3 Dutch strains from 2004 [24], and 20 Australian strains from the years 1995-1997 [25] of the RHD virus, analysing fragments of the gene encoding VP60. Also in the study performed by our team [26, 27], regarding the comparison of the N-terminal fragment of the gene encoding VP60 of five German strains [26] and 5 European strains (Hungarian, Czech, German) [27], homology was obtained at the level of 92-100%. Phylogenetic analysis of the very 'broad' material, namely 56 French strains of RHD from the years 1988-95 [28] and 104 French strains from the period 1993-2000 [29], based on the fragment of the gene encoding VP60, showed that they create 6 genogroups, and moreover, the study revealed that such distribution of strains in genogroups is probably also caused by the year and place of identification.

The above studies [1, 2, 9, 15-29] indicate that homology between the RHD virus strains oscillates at the level of 86-100%, which may suggest high similarity of their nucleotide sequences. The studies also suggest that even small differences recorded between RHDV sequences allow for grouping these strains into six genogroups which may, as presently reasoned, be conditioned with time and place of their reception. Such experiments have also proved the fact that there is a genetic distance between the strains identified in the first years (1987-1989) in Europe after the occurrence of the disease, and the strains identified nowadays (2001-2006) worldwide.

The aim of this study was to compare nucleotide sequences of 6 European strains of the RHD virus (Eisenhuttenstadt, Frankfurt, Rossi, V411, 24/89, 1447/96) received in the years 1989-2002, on the basis of the N-terminal fragment (510 nucleotides) and C-terminal fragment (320 nucleotides) of the gene encoding VP60, which shall allow to define the phylogenetic dependence between them. The results were compared to 5 strains (German FRG, Spanish AST89, Arab Saudi Arabia and American UT-01 and IN-05) with full recognised genome, as received in the years 1989-2005.

Material and Methods

The RHD virus

The virus was obtained from livers collected from experimentally infected rabbits with six European strains of the RHD virus coming from rabbits that naturally died in Germany (Eisenhuttenstadt, Frankfurt, Rossi), Hungary (24/89, 1447/96) and the Czech Republic (V-411), isolated in the years 1989-2002. The viral RNA was isolated from 30% of liver homogenates made in buffered solution of physiological salt (1 g of liver and 3 ml PBS) using the Total RNA set (A&A Biotechnology, Poland).

RT-PCR reaction

Complementary cDNA strain was received on the RNA matrix using reverse transcriptase (M-MLV Reverse Transciptase, Invitrogen, USA). The reaction used a specific anti-sense starter in concentration of 100 μM (Metabion GmbH, Germany), mixture of dNTPs nucleotides in concentration of 25 mM (Promega, USA), a reverse transcription kit M-MLVReverse Transciptase containing reverse transcriptase enzyme M-MLV RT, 5xbuffer RT-PCR, DTT 0.1M (Invitrogen, USA), RNase inhibitor RNase OUT (Invitrogen, USA), molecular biology water (Eppendorf, Germany) and RNA of the relevant strain of the RHD virus. At the beginning, the viral RNA was heated in the temperature of 65°C for 5 minutes, after which it was stored on ice until the time of adding the reactive mixture. For each of the RHD virus strains, the RT-PCR reaction was performed in two tests using two different starters. The RT-PCR reaction was performed in the T-gradient thermocycler (Biometra, Germany) using the following time-temperature profile: 25°C for 10 minutes, 37°C for 60 minutes, 95°C for 5 minutes, and 4°C for 1 minute. The resulting cDNA was stored at the temperature of 2-8°C for further analysis.

Starters

Starters were used that had been proposed by Guittre et al. [30] on the basis of full sequence of the genome of RHDV-FRG virus developed by Meyers et al. [30], and allowing for amplification of fragments of the gene encoding VP60 protein from the N- and C-terminal parts. Two pairs of starters were used:

- P1 (sense) 5'gagctcgagcgacaacaggc and P2 (antisense) 5'caaacacctgacccggcaac starters were used for the N-terminal part of the region encoding VP60 with the length of 510 nucleotides,

• P5 (antisense) 5'gcacctgcaagtccaatccg and P6 (sense) 5'acccagtcaggcaccaggctg starters were used for the C-terminal part of the region encoding VP60 with the length of 320 nucleotides.

PCR reaction

The PCR reaction was performed for each of the strains to amplify a fragment of the genome of the size of 320 nucleotides. In this reaction, starters were used in concentration of 10 µM each (Metabion GmbH, Germany), dNTPs mixture in concentration of 10 µM (Promega, USA), and polimerase kit comprising: 10xPCR buffer, Poly Taq DNA polimerase, and 10xmagnesium buffer (Promega, USA), molecular biology water (Eppendorf, Germany) and cDNA of the relevant RHD virus strain. The PCR reaction was performed in the T-gradient thermocycler (Biometra, Germany). The following time-temperature profile was applied: preliminary denaturation 94°C – 2 minutes, 35 cycles comprising: denaturations (94°C – 30 seconds), starter connection (53°C or 55°C – 1 minute), chain elongation (72°C – 2 minutes), and then final elongation 72°C for 5 minutes and cooling the reaction mixture to 4°C. The reaction products were stored at the temperature of 4°C for further analysis.

Agarose gel electrophoresis of PCR products

In order to visualise PCR products, electrophoresis was performed in 1.5% agarose gel (Prona, USA) coloured with etidine bromide (Fermentas, Lithuania) using molecular mass markers Gene O'Ruler 100 and Gene O'Ruler 50 (Fermentas, Lithuania). The electrophoretic separation was performed in 0.5 x concentrated TBE buffer at room temperature, with voltage of 100 V/cm of gel during 30 minutes using the electrophoresis kit (Bio-Rad, Germany). Archiving and interpretation of the results were made using the kit for visualisation in the UV light (Vilber Lourmat, France).

Preparative amplification, cleaning and preparation for sequencing of the analysed fragments of the RHD virus genome

After visualisation of the results of the PCR reaction, mass PCR was performed and electrophoretic separation of products in conditions identical to the aforementioned ones. Then, DNA was isolated in the gel using the Gel OUT kit (A&A Biotechnology, Poland), according to the manufacturer's procedure. Such isolated DNA was sent for automatic sequencing at the DNA Sequencing and Oligonucleotide Synthesis Laboratory of the IBB of the Polish Academy of Sciences in Warsaw.

Molecular phylogenetic analysis of the RHD virus sequence

The obtained nucleotide sequences of two fragments of the genome of six strains of the RHD virus were compared with one another (to obtain adjusted sequences, the sequences were cut to the length of 434 nucleotides and 295 nucleotides), and compared with homological sequences of strains with known full genome, collected from the Gene Bank database: FRG (M67473), AST89 (Z49271), Saudi Arabia (DQ189078), IN-05 (EU003578) and UT-01 (EU003582) strains. The analysis was performed in the DNAMAN software version 5.2.10 (Lynnon BioSoft, Canada).

Results

The obtained nucleotide sequences of the length of 434 nucleotides for the fragment of the N-terminal part of the region encoding VP60 of six strains (Eisenhuttenstadt, Frankfurt, Rossi, V411, 24/89, and 1447/96) of the RHD virus originating from Central Europe, and five strains (German FRG, Spanish AST89, Arab Saudi Arabia, and American UT-01 and IN-05) of full genome known were compared with one another. In the compared sequence of 11 strains analysed, genome mutations were recorded in the form of transition (80% of changes) and transversions (20%).

The phylogenetic tree created on the basis of molecular analysis of the fragment encoding the N-terminal part of the VP60 (Fig. 1), divided the 11 strains under study into two lines. The first line was formed by two American strains IN05 and UT01, with full genome known, and the German Rossi strain. Line two comprised 8 other strains, and was divided into two subgroups. The first subgroup was formed by the Spanish strain with full genome known AST89, and two German strains: Eisenhuttenstadt and Frankfurt. The second subgroup comprised German FRG strain, Hungarian 24/89 and 1447/96, Czech V411 and Arab Saudi Arabia strain, whereas genetic distance is observed for the Hungarian strain 1447/96 as compared to other 4 strains building this subgroup.

The homology tree created on the basis of molecular analysis of the fragment encoding the N-terminal part of the

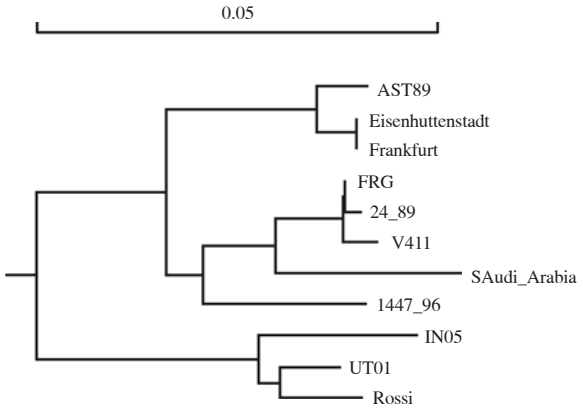

Fig. 1. Phylogenetic tree of 11 strains of the RHD virus created on the basis of molecular analysis of the fragment encoding the N-terminal part of VP60 polyprotein

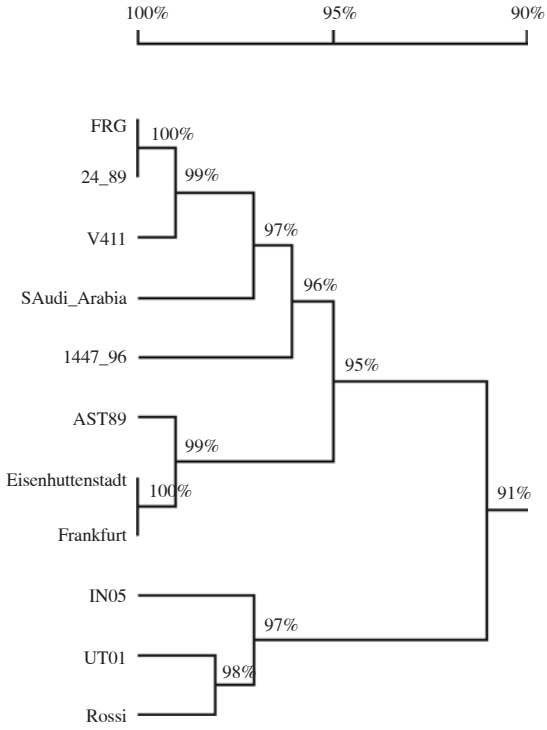

Fig. 2. Homology tree of 11 strains of the RHD virus created on the basis of molecular analysis of the fragment encoding the N-terminal part of VP60 polyprotein

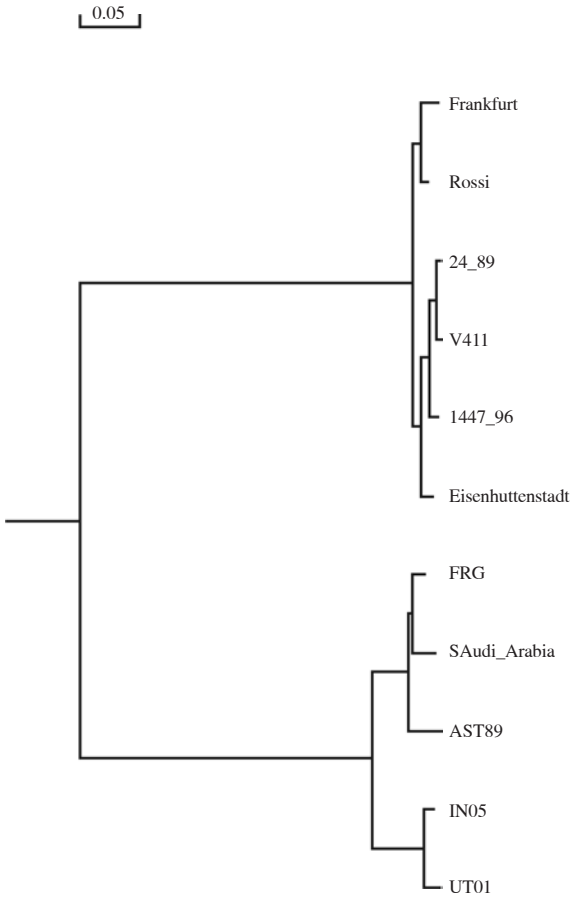

Fig. 3. Phylogenetic tree of 11 strains of the RHD virus created on the basis of molecular analysis of the fragment encoding the C-terminal part of VP60 polyprotein

VP60 (Fig. 2), divided the 11 strains under study analogically as the phylogenetic tree, into two groups with 91% of homology. Group one comprised American strains IN05 and UT01, and the German Rossi strain, whereas between UT01 and Rossi homology amounts to 98%, while as regards IN05, both such strains show 97% of homology. Group two comprises the remaining 8 strains forming two subgroups with 95% of homology. Subgroup one was formed by 3 strains: Spanish AST89 showing 99% of homology to two German strains with 100% homology: Eisenhutenstadt and Frankfurt. The other subgroup was formed by Hungarian strains 24/89 and 1447/96, German FRG, Czech V411 and Arab Saudi Arabia, showing 96-100% homology. The highest homology (100%) was recorded between the German FRG and Hungarian 24/89 strains, as regards to them the Czech V411 strain showed 99% homology. Saudi Arabia strain shows 97% homology to the group of three strains (FRG, 24/89, V411), while the Hungarian 1447/96 strain shows 96% homology to the remaining 4 strains from this subgroup.

The obtained nucleotide sequences for the fragment of C-terminal part of the region encoding VP60 with the length of 295 nucleotides from 6 strains of the RHD virus analysed presently and 5 strains of full genome were compared with one another. In the comparison created, genome mutations were recorded, and most frequently these were transitions (58% changes), while the remaining ones were transversions (42% of changes).

The phylogenetic tree created on the basis of molecular analysis of the fragment encoding the C-terminal part of the VP60 of 11 strains (Fig. 3), revealed the existence of two lines, within which two more subgroups were differentiated. The first line comprises 5 strains with full genome known, whereas the first subgroup is formed by American IN05 and UT01 strains, while the other – by German FRG and Arab Saudi Arabia strains, and the slightly distanced Spanish Ast89. Line two comprised the analysed Central European strains, whereas the first subgroup was formed by German strains Frankfurt and Rossi, while the other subgroup included Hungarian 24/89 and 1447/96 strains, and the Czech V411 strain, as well as slightly distanced to them German Eisenhuttenstadt strain.

The homology tree created on the basis of molecular analysis of the fragment encoding the C-terminal part of the VP60 (Fig. 4), divided the 11 strains under study analogically as the phylogenetic tree, into two groups. Group 1 comprises strains with full genome known, forming two subgroups with 89% of homology. Subgroup 1 comprised American UT01 and IN05 strains showing 98% homology to each other, while subgroup 2 was formed by the German FRG strain and Arab Saudi Arabia strain with 97% homology, as well as Spanish Ast89 strain showing 95% homology to the two previous ones. Group 2 comprised analysed strains from Central Europe, between which 96-99% homology was recorded, and which also created two subgroups with 96% homology. Subgroup 1 comprised German Rossi and Frankfurt strains showing 98% homology to each other. Subgroup 2 comprised Hungarian 24/89 and 1447/96 strains, Czech V411 strain, and German Eisenhuttenstadt strain, whereas the highest homology (99%) was observed between the Hungarian 24/89 and Czech V411 strains, to which 98% homology was recorded for the Hungarian 1447/96 strain, while the German Eisnehutenstadt strain showed 97% homology to the remaining strains in this subgroup.

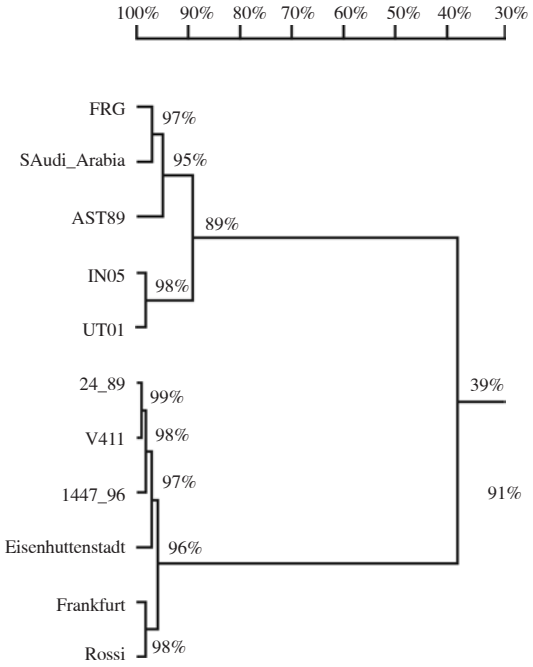

Fig. 4. Homology tree of 11 strains of the RHD virus created on the basis of molecular analysis of the fragment encoding the C-terminal part of VP60 polyprotein

Discussion

The genetic analysis of the six analysed strains of the RHD virus (Eisenhuttenstadt, Frankfurt, Rossi, V411, 24/89, 1447/96) from Europe, identified in the years 1989-2002, and 5 strains (FRG, AST89, Saudi Arabia, IN05, UT01) with full genome known, identified in various regions of the world in the years 1989-2005, on the basis of the generated phylogenetic and homology trees based on sequences encoding N- and C-terminal regions encoding VP60, indicated relatively small variability of strains within the analysed sequence of the N region (0-9%) and C region (1-11%). The results conform to the results obtained by other research teams [1, 2, 9, 15-29], where variability of the strains analysed was found at the level of from 0 to 14%. Homology obtained (91-100%) in the analysed N-terminal fragment of the gene encoding VP60 conforms to the results obtained in earlier studies by our team [26, 27] regarding the comparison of an analogical fragment in 5 German strains [26] and 5 European strains [27], where homology totalled 92-100%.

Among the 11 presently analysed strains, four came from the year 1989, and these included two strains with full genome known (German FRG, Spanish AST89), and two strains analysed that originated from Europe (German Eisenhuttenstadt, Hungarian 24/89). Three among the European strains came from the 1990s (Czech V411 – 1990, German Frankfurt – 1996, Hungarian 1447/96 – 1996), while the remaining ones were strains identified contemporarily, which included German Rossi strain and strains with full genome known: American UT01 (2001) and IN05 (2005), and Arab Saudi Arabia strain (2001). When analysing the phylogenetic and homology trees for the two fragments of the gene encoding VP60, it is hard to clearly explain grouping of the strains. Considering the N-terminal fragment of the gene encoding VP60 polypeptide, we notice that on one branch, there are strains identified in the years 2001-2005, but coming from two continents (American IN05 UT01 and German Rossi). Considering the year, one could explain the close distribution of the Spanish AST89 strain and German Eisenhuttenstadt strain, both identified in 1989, although this group also includes German Frankfurt strain from 1996, similarity of which may be explained by the site of identification. Such close distribution of the strains of German FRG, Hungarian 24/89 and Czech V411 might be explained with time of identification (1989-1990), while the inclusion in this subgroup of the Hungarian 1447/96 strain from 1996 may also be explained both by the year and place of identification. The presence of the Saudi Arabia strain in this group is, however, surprising as it can hardly be explained both by geographic proximity and coincidence in time. The distribution of strains originating from one country in one group is confirmed by the studies of Fitzner [1], who when comparing Polish strains (KGM, SGM, LUB, PD, MAL, ŻD, BLA) also obtained distribution of these strains in one group.

In the case of analysing the C-terminal fragment of the region encoding VP60, the strains analysed were not

distributed due to time or place of identification, but created two main groups: one included strains with full genome known, while the other – European strains analysed in this study. In these main groups, one may already see some time dependencies, such as even the distribution of the FRG strain in one subgroup with AST89, as the strains come from the same year. Such grouping is conformant to the results of the teams by Rasscharet [16], Boga [17] and Gould [18], where sequences of strains with full genome known were compared (German FRG, French SD, Czech V-351, Spanish AST89), identified in the years 1987-89. The 100% homology obtained in the N-terminal fragment of the gene encoding VP60 between the German Eisenhuttenstadt and Frankfurt strains probably results from the fact that they originate from one country, while analogical homology obtained between the German FRG strain and Hungarian 24/89 strain may result from the fact that they were identified in the same year. What seems surprising is the distribution of the Arab Saudi Arabia strain from 2001 with the German FRG strain (1989), Hungarian 24/89 (1989) and 1447/96 (1996) strains, and Czech V411 (1990) strain, as they are divided by both time and geographic distance. What is also surprising is the similarity of German Rossi strain of 2002 to American strains (IN05, UT01), which may result from similar time of their identification. In the N-terminal fragment analysed, such three strains (Rossi, UT01, IN05) created a separate group, showing 91% homology to the remaining 8 strains.

To conclude, one must state that 6 European strains analysed (Eisenhuttensatadt, Frankfurt, Rossi, V-411, 24/89, 1447/96) of the RHD virus and 6 strains (German FRG, Spanish AST89, American UT01 and IN05, and Arab Saudi Arabia) with full genome known, reveal high level of homology (89-100%). The level of homology is hard to interpret clearly and it cannot be stated whether it is related to the time or place of identification, which - as it is adopted - has been one of the elements allowing to divide the RHD virus into genogroups. This principally refers to European strains (Eisenhuttenstadt, Frankfurt, Rossi, V-411, 24/89, 1447/96), which are distributed into two groups in the case of the analysed N-terminal fragment of the gene encoding VP60 (group I – Eisenhuttenstadt (1989), Frankfurt (1996), 24/89 (1989) and 1447/96 (1996), and group II – Rossi (2002). The presently analysed European strains (Eisenhuttenstadt, Erfurt, Frankfurt, 24/89, 1447/96) included in Group I, group with strains (FRG, AST89, Saudi Arabia), while the analysed Rossi strain groups with American strains (UT01, IN05). In the event of an analysis concerning the C-terminal fragment, the analysed European strains (Eisenhuttenstadt, Frankfurt, Rossi, V-411, 24/89, 1447/96) form one group, while the comparative strains with full genome known form Group 2. The distribution obtained may result from differences in genetic construction, principally in the case of C-terminal fragment, and not the place (the same country) or year of their identification, although time distribution between the strains amounts from 3 to 16 years. The results obtained from the analysis of this particular fragment seem to confirm that it is C-terminal fragment of the gene encoding VP60 that is a very variable region in caliciviruses [31]. This is due to the fact that it is in this region that two hypervariable regions C and E are located, as differentiated by Neill [8] in the amino acid sequence. Furthermore, variability of most RNA viruses occurs during replication on the terms of mutations conditioned with RNA-dependent polymerase, whereas in RHD virus it is not yet documented, but it is suggested [32, 33]. It must also be added that high homology obtained in the analysed 11 strains (89-100%), originating from various regions of the world and obtained in the years 1989-2005, may result from the fact that the strains have evolved from other original strains, which is linked to RHD virus spread across the world and easiness of its transmission. It may be adopted that the homology obtained at the level of 89-100% and the division of 11 strains into 2 genetic groups may be linked to the fact that variability of RNA viruses (defined at the level of 1-2% during a year [34]), may cause changes to the genome, which differentiate RHD virus strains into subtypes.

Acknowledgements

This work was supported by grant No N308 03832/3662.

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