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# GENOMIC INTEGRATION OF HEPATITIS C VIRUS FRAGMENTS IN THE EUROPEAN RABBIT AND *LEPUS EUROPAEUS*: DETECTION AND CHARACTERIZATION

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# GENOMIC INTEGRATION OF HEPATITIS C VIRUS FRAGMENTS IN THE EUROPEAN RABBIT AND *LEPUS EUROPAEUS*: DETECTION AND CHARACTERIZATION

Tese de Candidatura ao grau de Doutor em Ciências Veterinárias, submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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“O Humano, Ser complexo, que muitas vezes se resume ao nada,
Independentemente disso, os pequenos fragmentos de qualquer coisa,
juntos ou separados, podem tornar-se num todo”

Eliane Silva, 2015

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**Paper 1**

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**Note 1**

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**List of Abbreviations**

| | |
|---|---|
| AFCS | Alliance for Cellular Signaling |
| ApoB | Apolipoprotein B |
| ApoE | Apolipoprotein E |
| BHV | Bat Hepacivirus |
| BSA | Bovine Serum Albumin |
| BT | Bovine Testicle |
| BVD/MD | Bovine Viral Diarrhea/Mucosal Disease |
| BVDV | Bovine Virus Diarrhoea Virus |
| CD81 | Cluster of Differentiation 81 |
| CDNA | Complementary DNA |
| CDS | Coding Sequences |
| CETI | Ethics Committee |
| CHV | Canine Hepacivirus |
| CI | Confidence Interval |
| CID | Collision Induced Dissociation |
| CLDN1 | Claudin 1 |
| $CO_2$ | Carbon Dioxide |
| DAAs | Direct-Acting Antivirals |
| Dano | Danoprevir |
| $ddH_2O$ | Double-Distilled Water |
| D-MEM | Dulbecco's Modified Eagle's Medium |
| DNA | Deoxyribonucleic Acid |
| DR | Domestic Rabbit |
| EASL | European Association for the Study of the Liver |
| EGFR | Epidermal Growth Factor Receptor |
| EIA | Enzyme Immunoassay |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ER | Endoplasmic Reticulum |
| EVEs | Endogenous Viral Elements |
| FBS | Fetal Bovine Serum |
| FCT | Fundação para a Ciência e Tecnologia |

| | |
|---|---|
| FITC | Fluorescein Isothiocyanate |
| GAGs | Glycosaminoglycans |
| GBD | Global Burden of Disease |
| GDD | Gly-Asp-Asp |
| GHV | Guereza Hepacivirus |
| H | Hare |
| HCC | Hepatocellular Carcinoma |
| HCV | Hepatitis C Virus |
| HCVuc | HCV uncharacterized |
| HHCV | Hare HCV-like virus |
| HIV | Human Immunodeficiency Virus |
| Huh | Hepatoma Cell Line |
| HVR | Hypervariable Region |
| ICNB | Instituto da Conservação da Natureza e da Biodiversidade |
| ICTV | International Committee on Taxonomy of Viruses |
| IDUs | Intravenous Drug Users |
| IEM | Immunogold Electron Microscopy |
| IFA | Immunofluorescence Assay |
| IFN | Interferon |
| IgG | Immunoglobulin G |
| IRES | Internal Ribosomal Entry Site |
| IUCN | International Union for Conservation of Nature |
| LDLR | Low Density Lipoprotein Receptor |
| LDs | Low-Density Lipoproteins |
| LH | Liver Homogenates |
| MAbs | Monoclonal Antibodies |
| MALDI | Matrix-Assisted Laser Desorption Ionization |
| MDBK | Mardin-Darby Bovine Kidney |
| MEGA5 | Molecular Evolutionary Genetics Analysis 5 |
| ML | Maximum Likelihood |
| MS | Mass Spectrometry |
| NANBH | Non-A Non-B Hepatitis |
| NC | Negative Controls |
| NCBI | National Center for Biotechnology Information |

| | |
|---|---|
| NJ | Neighbor-Joining |
| NPC1L1 | Niemann-Pick C1-like 1 |
| NPHV | Horse Hepacivirus |
| NS | Non-Structural |
| OCLN | Occludin |
| ORF | Open Reading Frame |
| P | Passage |
| p.i | Post Infection |
| PBS | Phosphate-Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PegIFN | Pegylated Interferon |
| PHH | Primary Human Hepatocytes |
| PHT | Postprandial Hypertriglyceridemic |
| qRT-PCR | Quantitative Real Time-Reverse Transcriptase-PCR |
| RBV | Ribavirin |
| RdRp | RNA-dependent RNA polymerase |
| RELIK | Rabbit Endogenous Lentivirus Type K |
| RHCV | Rabbit HCV-like virus |
| RHV | Rodent Hepacivirus |
| RNA | Ribonucleic Acid |
| RT-PCR | Reverse Transcriptase-PCR |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| SEQ ID | Sequence Identification |
| SMHL | St. Thomas Mixed Hyperlipidemic |
| SP | Signal Peptidase |
| SPP | Signal Peptide Peptidase |
| SRB1 | Scavenger Receptor Class B Type I |
| ssRNA+ | Single-Strand Positive-Sense RNA |
| SVR | Sustained Virological Response |
| TBS | Tris Buffered Saline |
| TfR | Transferrin Receptor |
| TJ | Tight Junctions |
| TOF | Time-of-Flight |
| UTR | Untranslated Region |

| | |
|---|---|
| UV | Ultraviolet |
| VLDL | Very-Low Density Lipoproteins |
| WBC | White Boold Cells |
| WHHL | Watanabe Heritable Hyperlipidemic |
| WHHLMI | Watanabe Heritable Hyperlipidemic Myocardial Infarction-Prone |
| WR | Wild Rabbit |

**List of International Units**

| | |
|---|---|
| % | Percentage |
| bp | Base pair |
| Da | Dalton |
| h | Hours |
| IU | International unit |
| kDa | KiloDalton |
| Log | Logarithm |
| min | Minutes |
| ml | Millilitre |
| mm | Millimetre |
| mM | Millimolar |
| nM | Nanomolar |
| ºC | Celsius degrees |
| U | Unit |
| w/v | Weight/volume |
| x *g* | Times gravity |
| µg | Microgram |
| µl | Microlitre |
| µm | Micometre |
| µM | Micromolar |

**ABSTRACT**

Cell foreign deoxyribonucleic acid (DNA) integration can occur non-naturally (transfections procedures) or naturally such as the integration of DNA from apoptotic bodies or by retrotransposition events. Viruses, the most abundant biological and diverse genetic entities on earth, capable of infecting the three major domains of life, can also become part of the genetic material of their host species and became endogenous viral elements (EVEs). These, could be derived from retrovirus, DNA or ribonucleic acid (RNA) viruses and there are several reports in animals and plant genomes. To date, just few EVEs related to the rabbit and hare genomes were reported in the European rabbit (*Oryctolagus cuniculus*) and European hare (*Lepus europaeus*), and rabbits have been used as a valuable animal model in research for the study of human diseases as they immune response to antigens is excellent and the well studied physiology of cells, sera, tissues and organs is more similar to humans than of mice and rats.

Hepatitis C virus (HCV) was discovered in 1989 and is the major causative agent of acute and chronic liver disease that can lead to hepatocellular carcinoma and death. HCV belongs to the *Flaviviridæ* family, genus *Hepacivirus*, and contains a single-strand positive-sense RNA genome. Only several years after HCV discovey some aspects of its life cycle were revealed due to a lack of an efficient cell culture system. HCV exhibits high genetic diversity and nowadays comprise 7 genotypes and 67 subtypes that are globally distributed. Moreover, HCV routes of transmission are strongly linked to the HCV high prevalence globally and are also related its high genetic diversity of the virus. The diagnosis of HCV infection is based on the presence of anti-HCV antibodies using enzyme immunoassays or the virus detection by conventional polymerase chain reaction (PCR) or real-time PCR. Nowadays, HCV treatment is based in combinations of direct-acting antivirals (DAAs) drugs (Sofosbuvir, Simeprevir and Daclatasvir) plus/not plus interferon-α and/or ribavirin. The human hepatoma cell line Huh-7 and clonal derivatives seems to be the most permissive cell lines for efficient RNA replication, resulted from manipulated genomic HCV strains, *in vitro*. However, other HCV-permissive cells like human non-liver origin, human liver-derived cells, and non-human cells have been described. Primary human hepatocytes (PHH) provide the closest *in vitro* model for the natural host cell of HCV and there are several studies reporting there use for wild type HCV isolates.

Although, as primary cells they rapidly lose their differentiation status influencing their use in HCV research. Small animal models with numerous experimental approaches have been taken to recapitulate parts of the virus life cycle and/or aspects of viral pathogenesis, but currently, chimpanzees remain the only model available for HCV research. To date the HCV origin remains unclear and HCV life cycle and pathogenesis are not enlightened processes. There have been recent descriptions on several studies reporting homologs of HCV detected in animals that could be a valuable contribute in these fields. Partially, our investigation also focused in the search for significant natural viruses reservoirs associated to animal or human diseases. Within this search we were led to hypothesize about the role of the European rabbit (*O. cuniculus*) and European hare (*L. europaeus*) as natural reservoirs of *Flaviviridae* or more precisely precursors of HCV-like viruses in this thesis.

Therefore, samples such as liver homogenates, body fluids, white blood cells, serum, liver homogenates inoculated in Mardin-Darby Bovine Kidney (MDBK) and/or bovine testicle (BT) cell cultures or DNA inoculated in MDBK cells, from wild and/or domestic European rabbits and/or from European hare were evaluated using different methods such as for PCR, reverse transcriptase-PCR (RT-PCR), mass spectrometry, immunofluorescence assay (IFA) and immunogold electron microscopy (IEM) assays. Endogenous HCV homolog fragments coding for HCV core, envelope glycoprotein's E1 and E2, protease NS2-3, serine protease NS3, NS4A, NS5A and NS5B specific proteins were detected in the tested animals genomes, showing their capacity to internalize and autoreplicate in MDBK and/or BT cell cultures. Moreover, when MDBK cells were inoculated with the rabbits or hare total DNA samples it was shown that HCV-like viruses are produced and that they are closely related to the HCV-1a and/or HCV-1b genotypes.

Additionally, preliminary work using synthetic fragments homologs to HCV and HCV wild type sera (HCV-1a, HCV-2c and HCV uncharacterized) samples were also inoculated in MDBK and/or BT cell cultures and evaluated by similar methods as of the animal samples. The results obtained suggests for the internalization and replication of the tested samples in the used bovine cell cultures.

The data presented in this thesis could contribute to understand the HCV origin and for a better understanding of the virus life cycle and pathogenesis, as well as contribute to the discovery of new antivirals and perhaps a vaccine production.

**Resumo**

O ácido desoxirribonucleico (ADN) pode ser inserido em células hospedeiras por via artificial (transfecção) ou naturalmente, por exemplo pela integração de ADN proveniente de corpos apoptóticos ou por eventos de retrotransposição. Os vírus são as entidades biológicas mais abundantes e geneticamente diferentes existentes na terra, sendo capazes de infetar os três principais domínios da vida. Estes podem ainda integrar o material genético das espécies hospedeiras normalmente referenciados como elementos virais endógenos (EVEs). Os EVEs podem ser derivados de retrovírus, vírus de ADN ou de ácido ribonucleico (ARN) e já foram descritos em genomas de plantas e de animais.

Até ao presente, muito poucas descrições têm associado os EVEs ao genoma do coelho e da lebre, havendo unicamente referências nas espécies de coelho Europeu (*Oryctolagus cuniculus*) e lebre Europeia (*Lepus europaeus*). O coelho tem sido também utilizado como espécie animal modelo relevante em investigação ligada a doenças humanas, uma vez que a sua resposta imunitária aos antigénios é excelente e, também porque a fisiologia das células, soros, tecidos e órgãos têm sido bem estudadas e assemelham-se em muitos aspetos aos seres humanos comparativamente aos ratos e ratazanas.

O vírus da hepatite C (VHC), descoberto em 1989,é o principal agente etiológico de doença hepática aguda e crónica que pode desencadear o desenvolvimento do carcinoma hepatocelular e morte. O VHC pertence à família *Flaviviridae*, género *Hepacivirus*, e possui um genoma de ARN de cadeia simples e polaridade positiva. Só apenas alguns anos após a sua descoberta foi possível desvendar alguns aspetos do seu ciclo de vida, principalmente porque a investigação na área do VHC tem sido dificultada pela escassez de sistemas de cultura de células eficientes. O VHC apresenta uma elevada variabilidade e consequente diversidade genética, e sabe-se que hoje engloba 7 genótipos e 67 subtipos que estão globalmente distribuídos. A sua transmissão está intrinsecamente associada à sua elevada prevalência a nível mundial e também relacionada à grande diversidade genética apresentada pelo vírus. O diagnóstico da infeção pelo VHC baseia-se na deteção de anticorpos anti-VHC através da utilização de ensaios imunoenzimáticos ou deteção do vírus utilizando o método convencional da reação em cadeia da polimerase (PCR) ou PCR em tempo real. O tratamento da

Hepatite C é baseado na utilização de combinações de drogas (Sofosbuvir, Simeprevir e Daclatasvir) antivirais de ação direta (DAAs) suplementadas ou não com interferão-α e/ou ribavirina. A cultura de células de linha do hepatoma humano Huh-7 e seus clones derivados têm-se apresentado como o melhor sistema de células permissivas à replicação eficiente do ARN, obtido de estirpes do VHC que foram geneticamente manipuladas, *in vitro*. No entanto, foram também descritos outros tipos de células permissivas ao VHC, tais como, células sem origem no fígado humano, outras derivadas do fígado humano e células sem origem humana. Os hepatócitos primários humanos (HPH) apresentam-se como o modelo *in vitro* mais próximo da célula hospedeira natural do VHC, e há vários estudos que descrevem a sua utilização para a multiplicação dos isolados de campo do vírus. No entanto, estas células primárias perdem muito rapidamente a sua capacidade de proliferação o que influencia a sua utilização na investigação do VHC. Já foram utilizados diversos modelos animais de pequeno porte para diferentes abordagens experimentais com o VHC, nos quais se conseguiram reproduzir partes do ciclo de vida do vírus e/ou aspetos da patogénese viral. No entanto, atualmente, os chimpanzés continuam a ser o único modelo disponível para a investigação do VHC. Até ao presente desconhece-se a origem do VHC e há fases e processos do seu ciclo de vida e patogénese por esclarecer. Alguns estudos mais recentes têm descrito a deteção de vírus homólogos ao VHC em diferentes espécies animais, podendo estes constituir um valioso contributo para esclarecimento destas áreas. Em certa medida, a nossa linha de investigação também tem como foco a procura de reservatórios naturais de vírus importantes associados a doenças animais ou humanas. A investigação apresentada nesta tese teve como base considerar a hipótese de qual poderia ser o papel do coelho Europeu (*O. cuniculus*) e da lebre Europeia (*L. europaeus*) enquanto reservatórios naturais de vírus da família *Flaviviridae* e mais especificamente, como precursores de vírus semelhantes ao VHC.

Para atingir os objetivos propostos, foram analisadas diversas amostras de coelho e de lebre, tais como homogeneizados de fígado, fluidos corporais, leucócitos, soro, homogeneizados de fígado inoculados em células epiteliais de rim de bovino (MDBK) e/ou testículo de bovino (BT), ou ADN total das duas espécies animais em estudo inoculados em células MDBK, utilizando diferentes métodos de diagnóstico e de investigação tais como, PCR, PCR via transcriptase reversa (RT-

PCR), espectrometria de massa, ensaio de imunofluorescência (IFA) e de imunomicroscopia electrónica (IEM). Foram detetados nos genomas dos animais testados, fragmentos endógenos homólogos ao VHC que codificam para proteínas do VHC, mais especificamente para as proteínas do “core”, glicoproteínas do invólucro E1 e E2, NS2-3 protease, NS3 serina-protease, NS4A, NS5A e NS5B, as quais demonstraram a capacidade de internalização e autoreplicação destes em células MDBK e/ou BT. Quando as células MDBK foram inoculadas com as amostras de ADN total do coelho ou lebre foi demonstrada a produção de partículas víricas semelhantes às do VHC as quais são muito próximas geneticamente aos genótipos VHC-1a e/ou VHC-1b.

Adicionalmente, foram realizados trabalhos de investigação utilizando amostras de fragmentos sintéticos homólogos ao VHC (regiões do “core”, E2 e NS4B) e de soros contendo estirpes de campo (VHC-1a, VHC-2c e VHC não-caracterizados) inoculadas em células MDBK e/ou BT, igualmente avaliadas por métodos de diagnóstico e de investigação semelhantes aos utilizados nos estudos anteriores. Os resultados preliminares obtidos sugerem a internalização e a replicação das amostras testadas nas culturas de células de espécie bovina utilizadas neste projeto.

Os resultados apresentados nesta tese poderão contribuir para o esclarecimento da origem do VHC e para um melhor esclarecimento do ciclo de vida e patogénese do vírus, assim como contribuir para a descoberta de novos antivirais e talvez na produção de uma vacina.

# CHAPTER 1

# STATE OF THE ART

# CELL FOREIGN DNA INTEGRATION, ENDOGENOUS VIRAL ELEMENTS, ENDOGENOUS VIRAL ELEMENTS INTEGRATED IN EUKARYOTIC GENOMES AND POSITIVE RNA VIRUSES

## 1. 1 - Cell Foreign DNA Integration

Foreign deoxyribonucleic acid (DNA) integration is one of the most intriguing cellular processes in molecular biology. For example, the development of several transfection procedures enable the DNA to enter into the cytoplasm, however its passage into the nucleus is mainly mediated by the cells machinery (1, 2) and after nucleus entry, the foreign DNA is large and rapidly degraded or diluted among subsequent cell divisions (3). Thus, under certain conditions, molecules containing an origin of replication such as a derived from viruses could persist for long periods as extrachromosomally replicating episomes, for example as expression of viral tumor antigens or in association with the nuclear matrix (4). In fact, foreign DNA integration process is not limited to laboratory transfections, integrative recombination studies or gene therapy studies for instance, as many natural cellular processes, such as the integration of DNA from apoptotic bodies (5) or retrotransposition events, give rise to *de novo* integration of DNA (6). Indeed, DNA integration may be seen as an ongoing natural process, which can be applied to artificially introduce modifications to cells in a genetic content. Moreover, viral DNA integration events appear to be identical in nature to any type of foreign DNA integration (4). Furthermore, in various natural and experimental scenarios, mammalian genomes become the targets for foreign DNA insertions, and so several DNA- and ribonucleic acid (RNA)-containing viruses are capable of integrating their genomes into the genomes of their host cells (7, 8).

## 1.2 - Endogenous Viral Elements

Viruses are the most abundant biological and diverse genetic entities on earth. They are environmentally ubiquitous and are capable of infecting Eukaryota, Bacteria and Archaea organisms as well as other viruses (9). Due to the infectious properties of viruses, which enable them to spread horizontally between individuals and across species, many viruses can also become part of the genetic material of their host

species, a process that is called endogenization (10). Moreover, viral insertions that result from the chromosomal integration of viral (DNA ) (or DNA copies of viral RNA in the host germ cells, which allows for vertical transmission and potential fixation in the host population, are called endogenous viral elements (EVEs) (11). EVEs were so far thought to be limited to retrovirus, endogenous retroviruses, but in a recent study (10) it was shown that EVEs could be derived from DNA (single or double strand) or RNA (positive or negative single strand and double strand) viruses too.

### 1.3 - Endogenous Viral Elements integrated in Eukaryotic Genomes

Most RNA virus EVEs identified to date derives from RNA viruses with negative-sense genomes or from RNA viruses that lack DNA intermediates (12-14). EVEs derived from flavivirus-related RNA viruses were first described in insects in 2004 (15). This description involves a gene sequence of an RNA virus that replicate using an RNA-dependent RNA polymerase integrated into the *Aedes* spp. mosquitoes genome (15). Recently, Katzourakis and Gifford described the presence of EVEs in animal genomes, including viruses derived from single-strand positive-sense RNA (ssRNA+) viruses, which has revolutionized the understanding of the processes and time-scale of viral evolution (11). More recently, EVEs that share a sequence similarity to ssRNA+ viruses of plants integrated into the genomes of insects have also been reported (16). However, EVEs derived from ssRNA+ viruses appear in extremely low copy numbers in the genomes; one genomic copy in the case of the *Reoviridae*, five in the case of the *Flaviviridae* and a small number in Potato virus Y were previously described (17). Moreover, EVEs from ssRNA+ viruses in honeybees were also described (18). More recently, a study that included EVEs related to ssRNA+ viruses also showed several EVEs related to negative ssRNA viruses, single and double strand DNA viruses, ssRNA-reverse transcriptase viruses and double strand-DNA-reverse transcriptase viruses in several Eukaryota genomes (10).

### 1.4 - Positive RNA Viruses

Positive RNA viruses are characterized by an ssRNA+ genome, which, upon virus entry and uncoating, functions as mRNAs and thus can be directly translated

into proteins by host cell machinery (19).Therefore, the genomic RNA could work as template for viral RNA replication. Following translation and processing of the viral polyprotein(s) and viral non-structural (NS) proteins, the viral RNA and host factors form membrane-associated replication complexes that carry out viral RNA synthesis (20, 21). The resultant progeny positive RNA strands can either initiate a new translation cycle or be packaged into virions that are subsequently released to infect naïve cells (20). Moreover, host factors participate in almost all or probably in all steps of ssRNA+ virus infection, including entry, viral gene expression, virion assembly and release (20). Furthermore, host factors are targeted by ssRNA+ viruses to modulate host gene expression and defenses (20).

## EUROPEAN RABBIT AND EUROPEAN HARE

### 1.5 - The European Rabbit (*Oryctolagus cuniculus*)

The European rabbit (*Oryctolagus cuniculus*), wild and domestic, are small mammals that belong to the Leporidae family within the order Lagomorpha. *Oryctolagus cuniculus* (*O. cuniculus*) which origin remains to the discovery of an oldest known rabbit fossil (6.5 million years) in the Iberian Peninsula (Andalusia, Spain) is the sole extant representative of the genus *Oryctolagus* (22). Two morphologically differentiated subspecies have been distinguished in 1914, the *O. cuniculus algirus* and *O. cuniculus cuniculus* respectively, and recently it was shown that they diverged ~1.8 million years (23, 24). Geographically, *O. cuniculus algirus* are located in the southwest and *O. cuniculus cuniculus* in the northeast of the Iberian Peninsula. *O. cuniculus cuniculus* expanded its range to the North towards France after the last glacial maximum (25), and still remains there. The rabbit distribution had a major impact when humans, during the Middle Ages, started transporting them to many other geographical areas (26) being found worldwide nowadays (e.g., Algeria; France; Gibraltar; Morocco; Portugal; Spain, Albania; Argentina; Australia; Austria; Belgium; Bulgaria; Chile; Croatia; Czech Republic; Denmark; Falkland Islands (Malvinas); Germany; Greece; Hungary; Ireland; Italy; Luxembourg; Namibia; Netherlands; New Zealand; Norway; Poland; Romania; Russian Federation; Slovakia; Slovenia; South Africa; Sweden; Switzerland; United Kingdom and United States).

On one hand, *O. cuniculus* is considered a pest in many countries and islands outside its natural range on the Iberian Peninsula, especially in Australia, South America and other European countries (27-33). On the other hand, during the 20th century, the number of rabbits on the Iberian Peninsula has declined >90% mainly because of viral diseases named myxomatosis, caused by a myxoma virus infection, and viral haemorrhagic disease; caused by the rabbit haemorrhagic disease virus and since 2011 the mortality rates are higher in Spain and Portugal due to the identified new variant of rabbit haemorrhagic disease virus that strongly affects domestic and wild rabbits populations (34, 35). Indeed, the European rabbit is currently classified in the International Union for Conservation of Nature (IUCN) in the red list of threatened species as near threatened (36).

The rabbits are valuable animal models in biomedical and fundamental research because they have many hereditary diseases (e.g. aortic arteriosclerosis, hypertension, hypertrophic cardiomyopathy and osteoporosis) common to humans and they have been commonly used in studies of toxicology, *in vitro* fertilization, embryology and organogenesis (37) as will be better exposed below.

### 1.6 - The European Hare (*Lepus europaeus*)

The European hare (*Lepus europaeus*), also known as brown hare, belong to the genus *Lepus* (order Lagomorpha, family Leporidae). The current Eurasian distribution of *Lepus europaeus* (*L. europaeus*) extends from the northern provinces of Spain, to introduced populations in the United Kingdom and southern regions of Scandinavia, south to northern portions of the Middle East, and has naturally expanded in the east to sections of Siberia (38). This species has been introduced as a game species extensively in several countries across the world [e.g., Argentina, Australia, Barbados, Brazil, Canada, Chile, Falkland Islands, New Zealand (North and South Island), Réunion, the United Kingdom and the United States] (38). The numbers of European hares have declined throughout Europe, with evidence of population decline beginning in the 1960s in association with the intensification of agricultural practices (39). In the last decades, a progressive decline in the hare population across Europe has also been associated to the occurrence of the European brown hare syndrome, a highly contagious disease caused by the

European brown hare syndrome virus, which nowadays is considered endemic in all European countries (40-42). Several countries have placed *L. europaeus* on their red list as near threatened or threatened (43) however the IUCN had placed it in the red list of threatened species as least concern (44).

### 1.7 - The European Rabbit and Hare Genomes and EVEs Integration

Comparative cytogenetic tools revealed that within the Leporidae family, which includes 56–60 recognized species depending on classifications, the chromosome numbers vary from 2*n* = 38 to 52 (45). Furthermore, when using available banded karyotypes for seven of the eleven Leporidae genera, a presumed ancestral karyotype with 2*n* = 48 chromosomes conserved with those of the European hare can be reconstituted (46). Moreover, it was shown that rabbit karyotype (2*n* = 44) differs from that of the European hare (2*n* = 48) by the presence of two centric fusions and that within Leporidae, karyotypes have either retained the presumed *lepus*-like ancestral state or have undergone various chromosomal rearrangements leading to chromosome patterns unique to each lineage (45, 47). Since the 1980s until today, several genetic polymorphisms in immunoglobulins, mitochondrial DNA and major histocompatibility complex class II genes, at a genetic variability level, have been described for rabbit and hare (48-54).

To date just few EVEs related to the rabbit and hare genomes were reported, and only to the European rabbit (*O. cuniculus*) and European hare (*Lepus europaeus*) genomes. The EVEs, of lentivirus origin, related to rabbit was first reported by Katzourakis *et al*, in 2007, and was named rabbit endogenous lentivirus type K (RELIK) (55). Moreover, it was also reported that these viruses integration were more than 7 million years old showing the first evidence for an ancient origin of the lentiviruses, however, later it was shown that these viruses diverged some 12 million years ago (56). Furthermore, the presence of the endogenous RELIK was newly reported in the rabbit genome in 2012 (57). In addition, a human retrovirus 5 was described as endogenous in the European rabbit genome (58). For the European hare, the identification of a RELIK orthologue in its genome with a minimum age of 12 million years for the lagomorph lentiviruses was described (59).

## 1.8 - Rabbits as Animal Models for the Study of Diseases

### 1.8.1 - Human Diseases

The biomedical research, basic science and applied research, is a huge effort that answers to medical questions, requiring the input and participation of many individuals with many different backgrounds and skills, such as, from life, biochemistry and physical sciences, among others. Moreover, they involve the investigation of the biological processes and the causes of diseases through careful experimentation, which involves observation, laboratory work, analysis and testing. The use of animals in laboratory experiments, following all ethical statements, presents an important contribute in this field, which can lead to the development of new preventions, therapies and cures for human and veterinary health.

The rabbit is a valuable animal model and research tool. For example, its immune response to antigens is excellent (45). The physiology of cells, sera, tissues and organs has been well studied and in several aspects is more similar to humans than mice and rats (45). They have small size, and are appropriate to collect adequate amounts of blood, milk or tissue samples for experiments or to be used for surgical experiments when compared with mice and rats, and the environment in which they are breed can be easily assayed (45). Firstly, rabbits were used for the production of antibodies and non-clinical safety studies for testing compounds, such as dermal tolerance test, pyrogen test, reproduction toxicity studies or ocular tolerance tests (60). Since 1980 the rabbit was introduced to function as an animal model for human diseases, using naturally mutant animals, genetic manipulated or through selective breeding (60). For example, in human hypercholesterolemia studies (translational medicine) it was shown that rabbits lipoprotein metabolism resembles of humans, when spontaneous hypercholesterolemic rabbits were used (61). Recently, a number of transgenic rabbits have been developed and contributed to the study of human cardiovascular diseases (62). Furthermore, rabbit models have been widely used for various purposes linked to human diseases studies as shown in Table 1.

**Table1.** Example of rabbit models applied to human diseases studies, adapted from (60).

| Human diseases | Rabbit models |
|---|---|
| **Lipid metabolism disorder** | |
| Hypercholesterolemia | WHHL* rabbit, WHHLMI* rabbit, cholesterol/ western diet-fed rabbit |
| Hyperlipidemia | SMHL* rabbit |
| Postprandial hypertriglyceridemia | PHT* rabbit |
| Metabolic syndrome | WHHLMI rabbit, SMHL rabbit, PHT rabbit |
| **Cardiovascular diseases** | |
| Atherosclerosis | WHHL rabbit, WHHLMI rabbit, cholesterol/ western diet-fed ordinary rabbit |
| Coronary atherosclerosis | WHHL rabbit, WHHLMI rabbit, cholesterol/ western diet-fed ordinary rabbits |
| Cerebral atherosclerosis | WHHL rabbit, WHHLMI rabbit |
| Myocardial infarction | WHHLMI rabbit |
| Cardiac function | WHHLMI rabbit, ordinary normal rabbits |
| Prolonged QT syndrome | Transgenic rabbits |
| **Other diseases** | |
| Xanthoma | WHHL rabbit, WHHLMI rabbit |
| Infectious diseases | New Zealand white rabbits, transgenic rabbits (Tuberculosis, Papillomavirus, Prion diseases) |
| Inflammatory response | Transgenic rabbits |

| | |
|---|---|
| Orthopedic surgery | Ordinary normal rabbits (Osteonecrosis, meniscal repair, autologous osteochondral transplantation, osteochondral defects) |
| Cardiovascular surgery | Ordinary normal rabbits (Spinal cord ischemia due to back-bleeding, dysfunction of aortic valve) |
| Transplantable Neoplasms | Ordinary normal rabbits (VX2 lung cancer, Kato sarcoma, Kondrateva osteogenic sarcoma, Brown-Pearce Carcinoma) |
| Keratopathy | WHHL rabbit, WHHLMI rabbit |
| Overactive bladder | WHHLMI rabbit |
| Hypacusia | WHHL rabbit |
| Chronic pancreatitis | WHHL rabbit |

*WHHL-Watanabe heritable hyperlipidemic; WHHLMI-Watanabe heritable hyperlipidemic myocardial infarction-prone; SMHL-St. Thomas' mixed hyperlipidemic; PHT-Postprandial hypertriglyceridemic.

### 1.8.2 - Infectious Diseases, RNA Viruses

During the 1980s and early 1990s several reports linked to RNA and DNA viruses, bacterial and fungal human infectious diseases using the rabbit as animal model were described (63). For example, the use of New Zealand White and the French Lop rabbits for studying the pathogenesis and immunology of rotaviruses infections were reported. In these studies it was demonstrated that the rabbit is susceptible to endogenous rotavirus strains that caused a disease that resembles the human disease epizootiologically and clinically (63-65). A non-respiratory animal model (New Zealand White rabbits) was developed to study the role of cellular immunity to parainfluenza virus type 3 infections and a T-cell response was developed, associated to the gut lymphoid tissue and in the bowel wall of PI-3 infected rabbits (66). Studies involving rabbits infected with human immunodeficiency virus (HIV) -1 were also described in this decade. One study showed that rabbits

infected with supernatant of H9 cells infected with HIV-1 produced detectable antibodies 2 weeks post infection and these animals remained seropositive for one year (67). Recently, a study designed to show whether molecular mechanisms are involved in the pathogenesis of fulminant hepatic failure in experimentally infected rabbits (as animal model) with rabbit hemorrhagic disease virus was described. These results support the rabbit usefulness, as animal model, in the investigation of potential novel therapeutically modalities aimed at neutralizing reactive oxygen species and hepatocyte growth inhibitors or enhancing hepatocyte responsiveness to mitogens (68). More recently, Cheng and colleagues (69), following the identification of hepatitis E virus from rabbits (70), accessed the possibility of using rabbits as a non- human primate animal model for hepatitis E virus infection and vaccine evaluation (69). They have concluded that the rabbits may serve as an alternative model for hepatitis E virus infection and vaccine evaluation of selected strains, with the appropriate viral dosages and use of the intravenous route for virus inoculation (69).

## HEPATITIS C VIRUS

### 1.9 - Disease

Hepatitis C virus (HCV), a positive RNA virus with a +ssRNA genome is the cause of a contagious liver disease that is characterized by two phases, an acute and chronic phase. The acute hepatitis C infection involves a short-term illness that develops within the first six months after a person has been exposed to the HCV virus. The acute hepatitis C infections can lead to chronic hepatitis. Chronic hepatitis C infection involves illness that is long-term and happens when the HCV virus is not cleared from the infected person. The infection may last for the duration of the person's lifetime, leading to serious complications in the liver, such as cirrhosis or hepatocellular carcinoma (HCC), or even result in death of the host (World Health Organization).

### 1.10 - Discovery

Throughout the 1970s and early1980s clinicians and epidemiologists found that when a substantial number of patients were screened for hepatitis viruses they

were not related with hepatitis A or hepatitis B viruses. Consequently, a new form of hepatitis was achieved, the non-A non-B hepatitis (NANBH). At this time chronic NANBH associated with blood transfusion and therapy with plasma-derived blood products was described (71-75). However, this new clinical entity remained unclear. Later, in 1989, Choo *et al*. (Chiron Corporation) addressing molecular biological methods isolated a complementary DNA clone (5-1-1) from a blood-borne non-A, non-B viral hepatitis genome that was shown to be derived from a new "flavi-like" virus and was termed the "hepatitis C virus" (76). For this, large volumes of chimpanzee sera with high infectious titers of NANBH were extracted for RNA and DNA isolation. From RNA, complementary DNA (cDNA) was performed and then inserted into a bacteriophage expression vector lambda gt 11, resulting in a random-primed cDNA library with millions of clones (76). Then, using a blind immunoscreening approach, the cDNA library was screened using plasma from NANBH infected patients which resulted in the isolation of a positive clone called 5-1-1 with ~10,000 nucleotides derived from an RNA molecule found only in NANBH-infected samples. The southern blot analysis indicated that the clone was not a host gene derived from the chimpanzee or human genome and that it only bounded to antibodies in NANBH infected patients (76). A larger clone from the same library, clone 81, was also investigated and confirmed the potential viral origin of clone 5-1-1 (76). This work leaded to the discovery of the "hepatitis C virus".

## 1.11 - Taxonomy

### 1.11.1 - Genus Classification

The *Flaviviridae* family is classified by the International Committee on Taxonomy of Viruses (ICTV), http://ictvdb.bio-mirror.cn/Ictv/fs_flavi.htm) as a composite of tree genus of assigned viruses, the *Flavivirus*, *Pestivirus* and *Hepacivirus*, with the HCV (a virus that infect humans) considered the only member of the *Hepacivirus* genus. However, recently, the *Flaviviridae* family was for the first time described as including four genera, *Flavivirus*, *Pestivirus*, *Hepacivirus*, including the GBV- B viruses (77) and others viruses that are able to infect animals, and *Pegivirus* (78) (Figure 1.1), due to the description of HCV-like viruses, previously termed *Hepacivirus*, that were able to infect dogs, horses, bats and rodents (79-82) and viruses that infect bats and rodents homologous to the genus *Pegivirus* (81, 82).

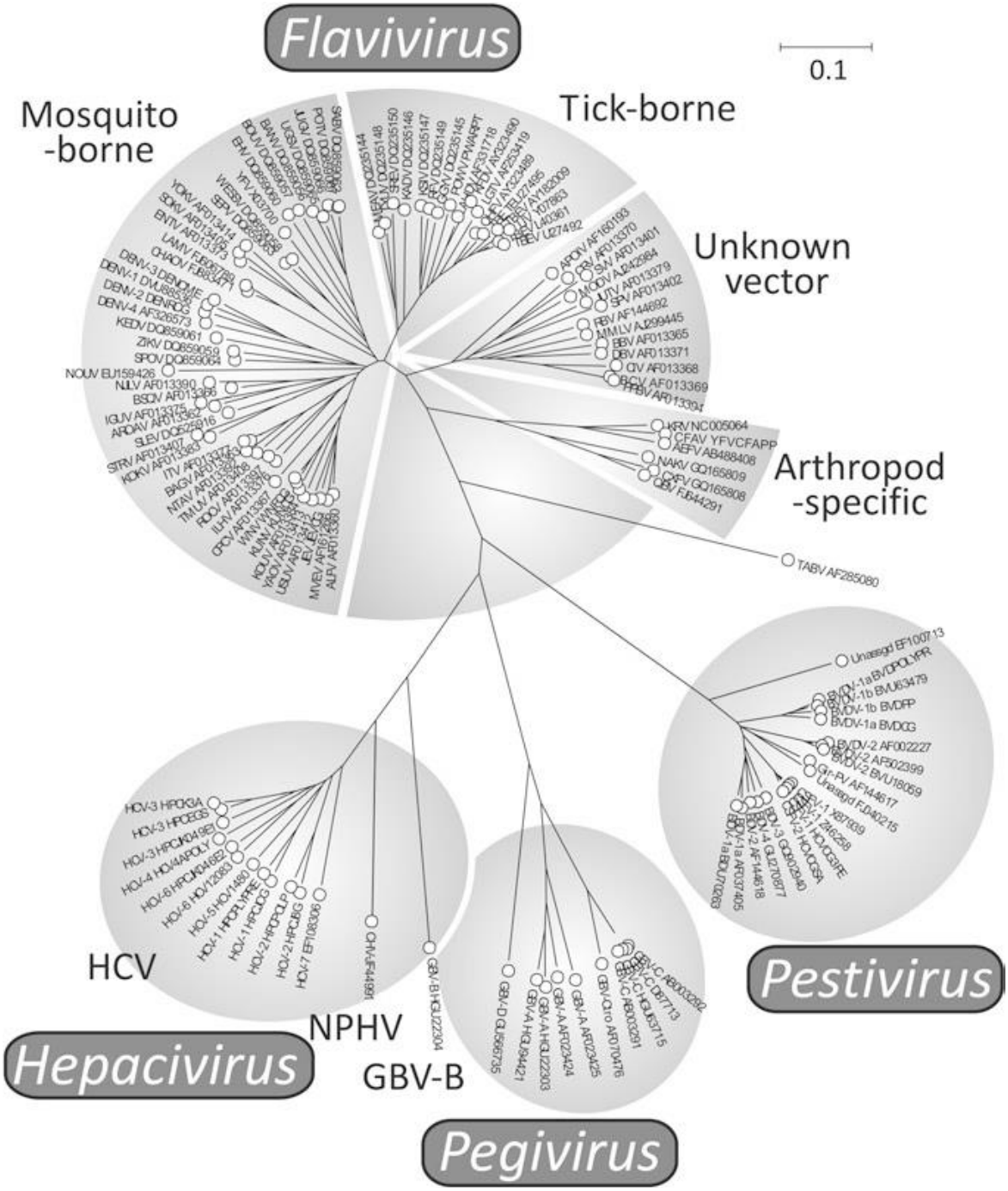


**Figure 1.1.** *Flaviviridae* division into four genera. Phylogenetic tree of the family *Flaviviridae* showing its primary division into four genera (78).

### 1.11.2 - Genotypes and Subtypes Classification

The first nearly complete genome identification of HCV occurs in 1989 when the virus was discovered and named HCV for the first time. Soon after, genetic

diversity of the virus was demonstrated when isolates from different individuals or countries were screened. Later, in 2005, this variation leaded to genotypes and subtypes assignment in a consensus classification and nomenclature system (77). Moreover, for genotype and subtype assignments four rules were established: one or more complete coding region sequence(s) should be determined; at least three epidemiologically unrelated isolates; a phylogenetic group distinct from previously described sequences; and exclusion of intergenotypic or intersubtypic recombination, whether the components were classified or not (77, 83). Furthermore, updates and changes of the rules for genotype/subtype assignments related to subtype names; provisional genotypes and subtypes; recombinant and other forms; and proposals for new genotype/subtype assignments were recently proposed (83).

In the last years the molecular biology technology approaches, such as polymerase chain reaction (PCR) and sequencing methods (such as dideoxy sequencing and pyrosequencing), contributed to an important increase of the vastly encountered HCV genetic variation in disease and treatment approaches. A large number of sequences became available in sequence databases (such as, National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), Los Alamos HCV Sequence Database (http://hcv.lanl.gov/content/index), European HCV database (euHCVdb) (https://euhcvdb.ibcp.fr/euHCVdb/), Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/)), and several of them contributed for the new described expanded classification of HCV into 7 genotypes and 67 subtypes (83) (Figure 1.2). Additionally, it was also shown that HCV strains belonging to different genotypes differ at 30 to 35% of nucleotide sites and strains that belong to the same subtype differ at <15% of nucleotide sites with all genotypes having a similar replication cycle in the host and capable of causing asymptomatic and chronic infections (83)

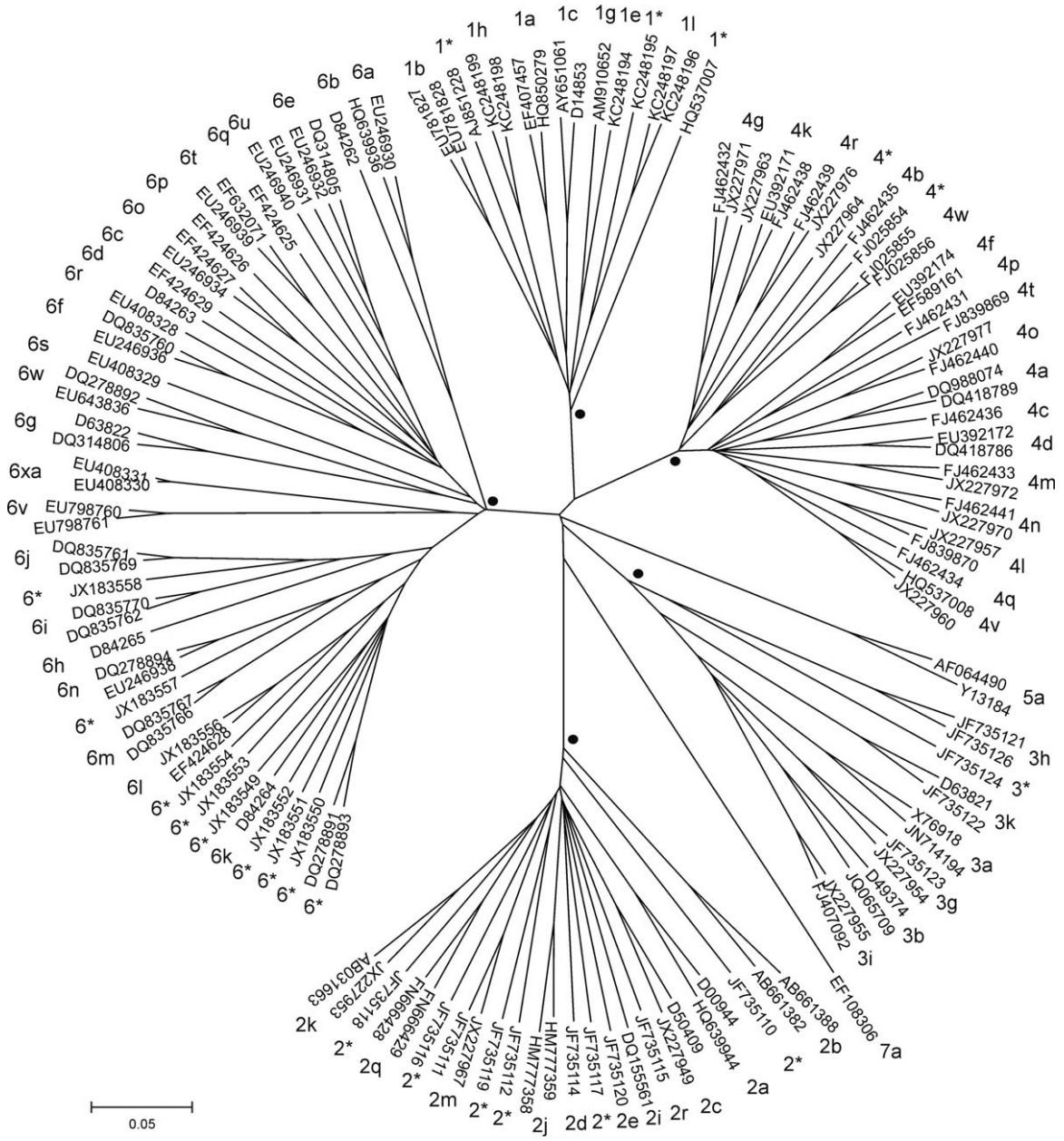


**Figure 1.2.** Phylogenetic tree of 129 representative complete coding region sequences. Up to two representatives of each confirmed genotype/subtype were aligned (together with a third extreme variant of subtypes 4g and 6e) and a neighbor joining tree constructed using maximum composite likelihood nucleotide distances between coding regions using molecular evolutionary genetics analysis 5 (MEGA5). Sequences were chosen to illustrate the maximum diversity within a subtype. Types are labeled by accession number and subtype (*unassigned subtype). For genotypes 1, 2, 3, 4, and 6, the lowest common branch shared by all subtypes and supported by 100% of bootstrap replicates (n = 1,000) is indicated by ●(83).

### 1.12 - Structure and Genome Organization

HCV are small (55-65nm in size) enveloped viruses and the viral particle consists of a core of genetic material (RNA), surrounded by an icosahedric protective shell of protein, and further encased in a lipid envelope of cellular origin (84) (Figure 1.3A). HCV contains a ssRNA+ genome of ~9,600 nucleotides and is composed of a 5′ untranslated region (UTR), a long open reading frame (ORF) encoding a polyprotein precursor of ~3000 amino acids, and a 3′ UTR (85, 86) (Figure 1.3B). The 5`and 3`UTR regions are highly conserved RNA structures important for genome replication and protein translation. The polyprotein is composed by several proteins that are necessary for viral particle attachment and entry into the host cells, including structural (core and envelope (E) glycoprotein's E1 and E2) and NS (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (86-88) (Figure 1.3B).

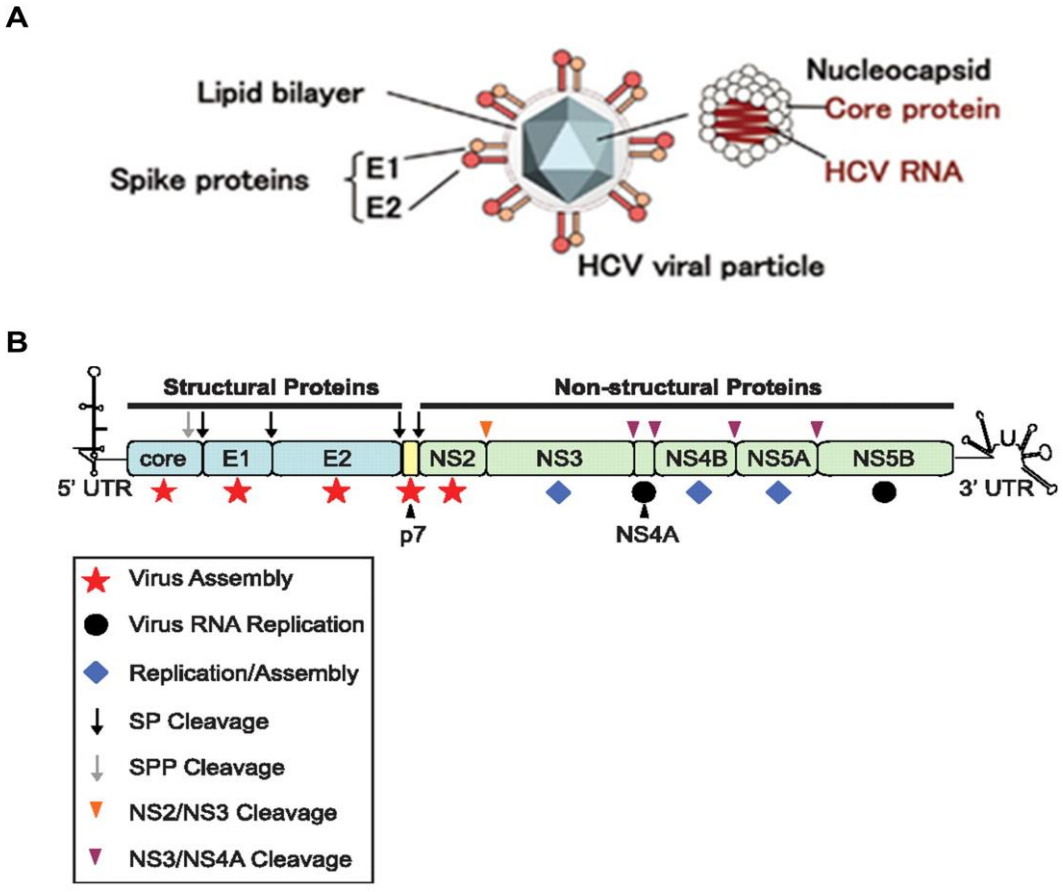


**Figure 1.3.** Schematic representation of the HCV viral particle and genome structures, adapted from (89, 90)**.** (A) HCV viral particle; the HCV core protein

interacts with viral genomic RNA to form the nucleocapsid. Two membrane-associated envelope glycoproteins, E1 and E2 are embedded in a lipid envelope which is derived from the host. (B) The single open reading frame encode a polyprotein with 10 viral proteins that are divided into the structural (core, E1 and E2; in blue) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B; in green) proteins. The small peptide p7 is currently unassigned into either category (yellow). The open reading frame is flanked by 5`UTR and 3`UTR regions that are involved in both replication and translation. Following translation, the viral polyprotein is processed by two cellular protease activities, signal peptidase (SP; black arrow) and signal peptide peptidase (SPP; gray arrow), to yield core, E1, E2, and p7. The NS2-NS3 autoprotease (orange arrowhead) and the NS3 serine protease as a complex with NS4A (purple arrowhead), generate mature proteins from the NS2-NS5B region. Core, E1, and E2 provide physical components of the virion. The p7 and NS2 are essential for virus assembly (red stars). NS4A and NS5B (black circles) are critical for viral RNA replication. NS3, NS4B, and NS5A have dual functions in viral RNA replication and particle production (blue diamonds).

### 1.13 - Life Cycle

HCV was discovered in 1989 (76), but only several years latter it was possible to highlight some aspects of its life cycle, mainly because HCV research has been hampered by the lack of efficient cell culture systems. However, the generation of subgenomic HCV replicons (91), the HCV pseudoparticle approach (92) and infectious HCV culture systems (93-95) were crucial developments that assisted to understand and obtain a more complete view of HCV life cycle. HCV infects and replicates in liver hepatocytes of infected patients and so cell culture systems approaching the hepatoma cells lines, such as Huh-7 cell line (96, 97) seemed to be the most suitable to understand the virus life cycle, however, this cell line lacks some features of the hepatocytes (98). Indeed, nowadays just hypothetical models for the virus life cycle are suggested and so HCV life cycle stills an active research area.

#### 1.13.1 - Entry

HCV entry is a complex and multi-step process requiring a set of entry proteins (Figure 1.4). During a primary infection, HCV particles are transported by the

blood stream, cross the fenestrated endothelium of the liver sinusoids and in the space of Disse, the virions have direct contact with the basolateral surface of hepatocytes allowing them to interact with factors and receptors present in this surface (99).

Initial attachment of HCV virions into hepatocytes, depending on virion density, could be mediated by the heparan sulfate proteoglycan syndecan-1 or syndecan-4 (100, 101) or by the scavenger receptor class B type I (SRB1) (102, 103). However, more recent data suggest that apolipoprotein E (ApoE), rather than HCV glycoproteins themselves, could be involved in this initial contact (103, 104). Also the low density lipoprotein receptor (LDLR) is thought to be involved in early phase of HCV entry (105), although this finding remains somehow controversial because HCV-LDLR interaction seems to involve a non-productive entry pathway that can potentially lead to viral particle degradation (106). HCV cell entry requires also others major essential cellular factors, receptors or co-receptors, like human cluster of differentiation 81 (CD81) (107), claudin 1 (CLDN1) (108) and occludin (OCLN) (109). SRB1 interacts with HCV glycoprotein E2 and lipoproteins, suggesting that it could be the first entry factor that interacts with the virion after initial cell attachment, through its lipid transfer activity, it could modify the lipid composition of the lipoprotein moiety of the virion, exposing the CD81 binding site on E2 glycoprotein (110). However, it was described that reduced dependency on SRB1 of hypervariable region 1 (HVR1), a part of E2 glycoprotein, in deleted mutant viruses could unmask the CD81 binding site of E2 glycoprotein (111, 112). Independently of these observations, HCV virions seem to interact with CD81 after SRB1 binding. CD81 belongs to the family of tetraspanins and is ubiquitously expressed, except in red blood cells and platelets, being definitely a key player in the HCV life cycle (113). The CD81 actively promotes infection upon HCV binding by triggering signaling cascades that are important for virus entry (114). Furthermore, CD81 is highly dynamic at the cell surface and is enriched in membrane areas that form stable platforms, which are in permanent exchange with the rest of the membrane, and the balance of these dynamic exchanges in the cell membrane are essential to the process of HCV entry (99, 115, 116). The CLDN1, another essential entry factor has also been shown to interact with CD81 to form a co-receptor complex involved in HCV entry events (115, 117, 118) and CD81- CLDN1 association appears to be regulated by multiple

signaling pathways (99). Furthermore, the viral particle might also potentially interact with CLDN1 (119). Additionally to CLDN1, another tight junction protein (OCLN) is also an essential HCV entry factor (109), that seems to play a role at the late entry step (120, 121), although the precise role of this protein in the HCV life cycle remains unclear. Recently, the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) was identified as an additional HCV entry factor when was related to the high cholesterol presence in the HCV particles and transferrin receptor 1 as also been described to be involved in HCV entry, however the precise roles of them remain to be clarified (122).

Finally, HCV particle is internalized by clathrin-mediated endocytosis and fusion takes place in early endosomes.

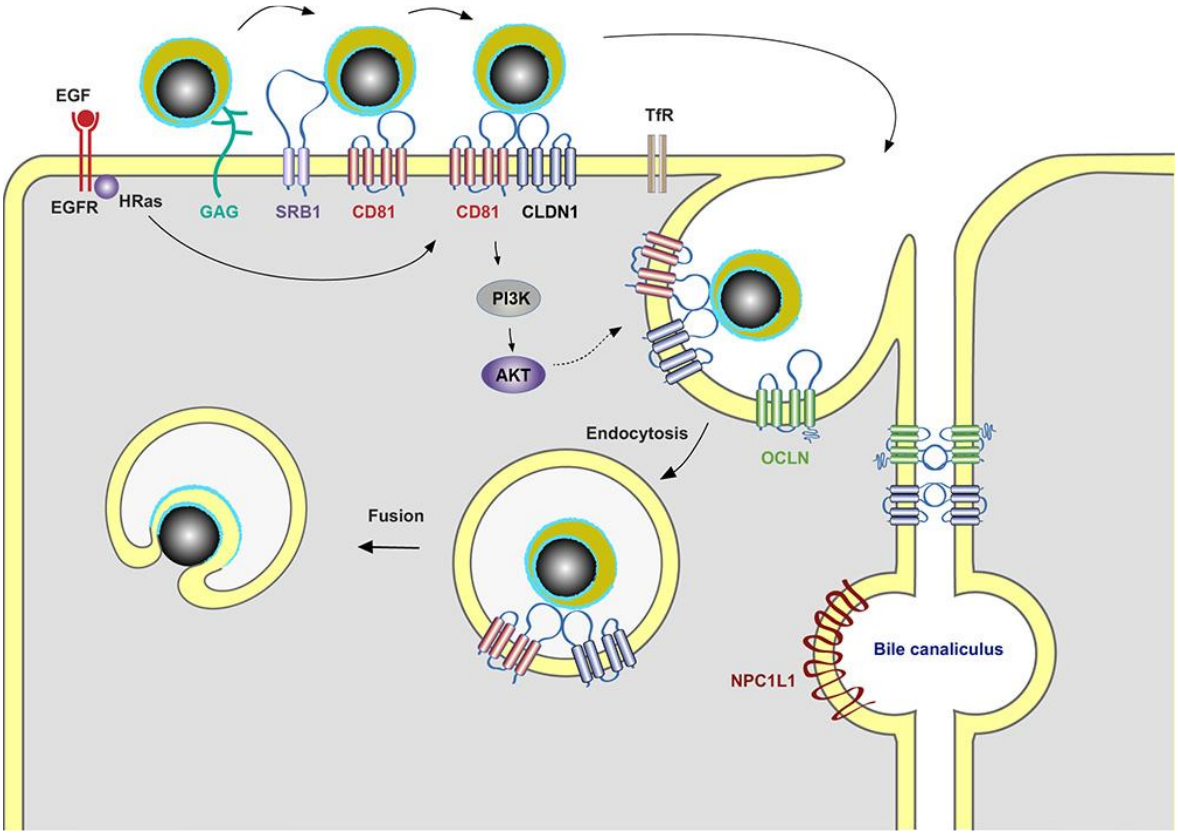


**Figure 1.4.** HCV entry. The HCV particle initiates its life cycle by binding to glycosaminoglycans (GAGs) and SRB1. Then the virus follows a complex multistep process, involving several specific cellular entry factors, such as SRB1, CD81, CLDN1 and OCLN, epidermal growth factor receptor (EGFR), transferrin receptor (TfR) and NPC1L1, as well as signaling proteins. After binding to several

components of the host cell, HCV virion is internalized by clathrin-mediated endocytosis and fusion takes place in early endosomes (99).

### 1.13.2 - Translation and Replication

After infection of a cell, the positive-strand RNA genome of HCV directly serves as the template for translation in the cytosol (123). The 5` UTR region contains an internal ribosomal entry site (IRES), which initiates translation of the HCV genome into a single polyprotein (123). Viral and host encoded proteases process the viral polyprotein into the 10 mature proteins, 3 structural proteins (core, E1, E2) and 7 non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (124). Signal peptidase and signal peptide peptidase mediate cleavage of the core, E1 and E2 proteins, and the p7/NS2 junction (99). NS3 mediates cleavage of NS4A from itself and NS4B, after which NS4A associates with the N-terminus of NS3 resulting in a NS3/4A protease complex (99). This complex can then cleave at the NS4B/5A and NS5A/5B junctions, whereas the cleavage between NS2 and NS3 is mediated by the NS2 cysteine protease whose function is strongly enhanced by the N-terminal one-third of NS3 (99). After translation, the HCV proteins are associated with membranes resultant from the endoplasmic reticulum (ER) (Figure 1.5). Replicon machinery, constituted by viral proteins, NS3/4A, NS4B, NS5A and NS5B, replicates the positive sense RNA genome through a negative strand intermediate (125). Furthermore, the viral RNA-dependent RNA polymerase (NS5B protein) is the key enzyme of RNA synthesis (99). Nascent RNA genomes are translated to produce new viral proteins, which could work as new or additional RNA templates for further RNA replication and are progressively assembled to form infectious virions (99). Moreover, microRNA 122 (miR-122), a liver-specific microRNA that is highly expressed, acts in an unusual manner to stimulate accumulation of HCV RNA by interacting with the 5′ UTR region of the viral genome (126-128).

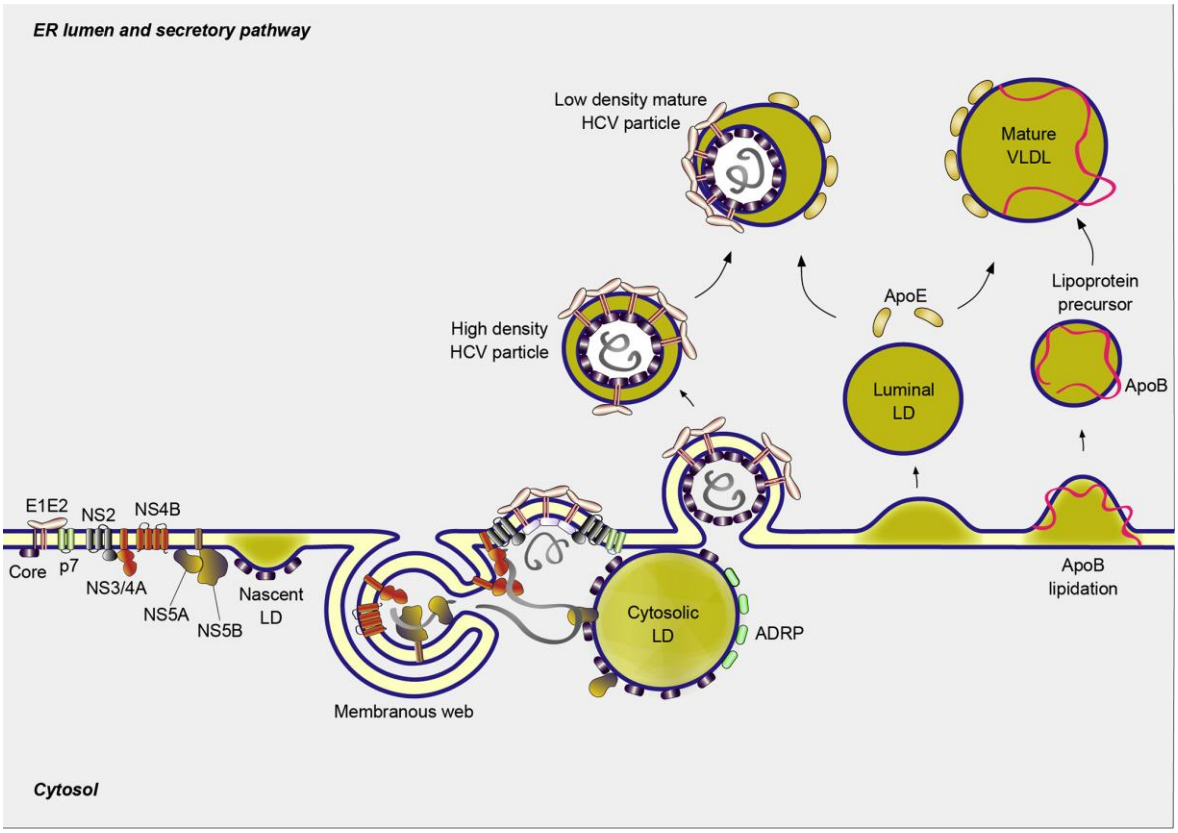


**Figure 1.5.** HCV replication and assembly. Upon cleavage of the polyprotein, HCV non-structural proteins form the replication complex in association with cellular factors lead to the formation of membranous web where replication takes place. After cleavage of its C-terminus, the core protein is loaded against low-density lipoproteins (LDs). The junction between core-loaded LDs and the replication-complex-rich ER membranes is the site of virion assembly. Newly replicated viral genomes are transferred to the assembly sites via NS3/4A or NS5A proteins, and NS2 protein and p7 protein connect replication complexes and core proteins to the glycoproteins. The next steps in HCV virion morphogenesis are tightly associated to the metabolism of very-low density lipoproteins (VLDL) assembly. However, the details of the intersection between HCV assembly and VLDL biogenesis remain unclear. It has been demonstrated that HCV particle contains ApoE and neutral lipids, but how these components are acquired during HCV morphogenesis remains poorly understood. This figure represents a hypothetical model (99).

### 1.13.3 - Assembly and Release

Currently, all structural and non-structural HCV proteins, alone or in complexes between some of them were demonstrated to be involved in the virion assembly process, when they interact within host cells, in pathways that occur in the cytosol involving lipid droplets, LDs, VLDL, apolipoproteins and receptors, for instance (Figure 1.5). One peculiar feature of HCV particle assembly when compared with other members of the *Flaviviridae* family is the implication of the viral non-structural proteins in this process (99). A major component of the viral particle is the core protein, which interacts with the genomic RNA to form the nucleocapsid (84, 89). After synthesis on ER membranes and cleavage by the signal peptide peptidase, the core protein homodimerizes and is transferred to the cytosolic LDs (129, 130). The envelope glycoprotein complex is also a major component of the viral particle and it was described that the NS2 protein interacts with envelope glycoproteins (E1 and E2) and the p7 protein and that these interactions are essential for the migration of the glycoproteins heterodimer at the viral particle assembly site (131). The C-terminal domain III of NS5A protein also involved in the viral particle assembly is essential for NS5A interaction with the LD-bound core protein (132), an essential step in HCV assembly. Moreover, NS3/4A, NS4B and NS5B proteins have also been described as implicated in virus assembly (133, 134). On the late stage of assembly, the maturation and release of HCV particles was shown to be associated with the VLDL pathway (133, 135). Furthermore, apolipoproteins such as apolipoprotein B (ApoB) can also be found in association with HCV particles; however the functional importance of ApoB for assembly and infectivity is controversial (136). After assembly and budding in the ER, HCV particles are released from cells by transit through the secretory pathway (137).

## 1.14 - Epidemiology

HCV infections are a major global health burden and a leading cause of death and morbidity (138). Moreover, persistent HCV infection is associated with the development of liver cirrhosis, liver failure, HCC and death (139). HCV is now the most common cause of death in HIV positive patients on highly active antiretroviral therapy (140). While the incidence rate of HCV infection is apparently decreasing in

the developed world, however deaths from liver disease secondary to HCV infection will continue to increase over the next 20 years (141). Furthermore, HCV routes of transmission are strongly linked to the HCV high prevalence globally and is also related to the high genetic diversity of the virus Recently, an estimate of the disease burden showed an increase in seroprevalence of 2,8% in the last 15 years, equating to >185 million infections worldwide (142). As described above, HCV exhibits high genetic diversity, comprising 7 genotypes and 67 subtypes. Subtypes 1a, 1b, 2a, and 3a, considered epidemic subtypes, are widely distributed across the world and are associated with a large proportion of HCV infections in high income countries (143). This occurred, probably due to the rapid spread of the virus before HCV discovery, when NANBH was associated with infected blood, blood products (72, 75) or by injecting drug users (144),for instance. Moreover, other HCV genotypes are considered endemic strains, as they are comparatively rare and have circulated for a long period of time in more restricted regions, such as genotype 1 and 2 endemic strains primarily in West Africa, genotype 3 in South Asia, genotype 4 in Central Africa and the Middle East, genotype 5 in Southern Africa and genotype 6 in South East Asia (143, 145-147). Relatively to genotype 7, few infections have been described in Canada and in Central Africa (148, 149).

A recent study involving global distribution and prevalence of HCV genotypes was reported (143). Considering HCV genotype prevalence published data between 1989 and 2013 combined with overall HCV prevalence estimates from the Global Burden of Disease (GBD) project was accessed; 1,217 studies were included in the analysis, representing 117 countries and 90% of the global population (143). Moreover, HCV genotype 1 was shown to be the most prevalent worldwide (83.4 million cases, 46.2% of all HCV cases), genotype 3 being the next most prevalent globally (54.3 million, 30.1% of all HCV cases); genotypes 2, 4, and 6 responsible for a total 22.8% of all cases and genotype 5 comprising the remaining <1% (Figure 1.6) (143).

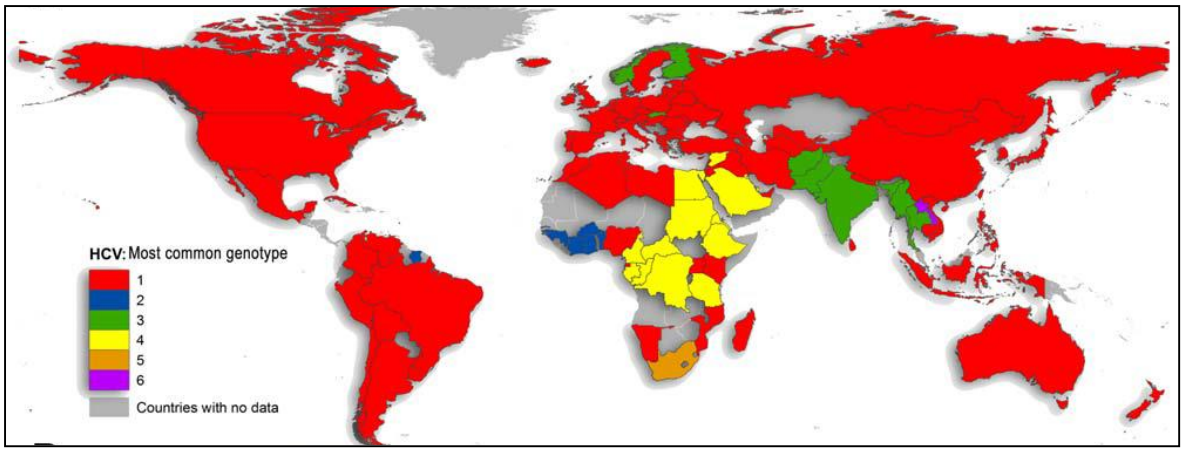


**Figure 1.6.** HCV prevalence in countries by the majority genotype found across all associated studies (143).

Currently, there are just a few of isolates identified for genotype 7, the reason for this genotype to be not included in the described study (143). Furthermore, relative prevalence of each HCV genotype by GBD region and across all virus samples by country could be observed in figure Figure 1.7. and 1.8, respectively. Additionally, in Figure 1.8 considering Portugal (4 studies, Table 1 (143)) is illustrated that genotype 1 shown a relative prevalence between 50-75%, genotype 2 0.1-10%, genotype 3 25-50%, genotype 4 10-25%, genotype 5 0.1-10% and genotype 6 < 0.1% (143).

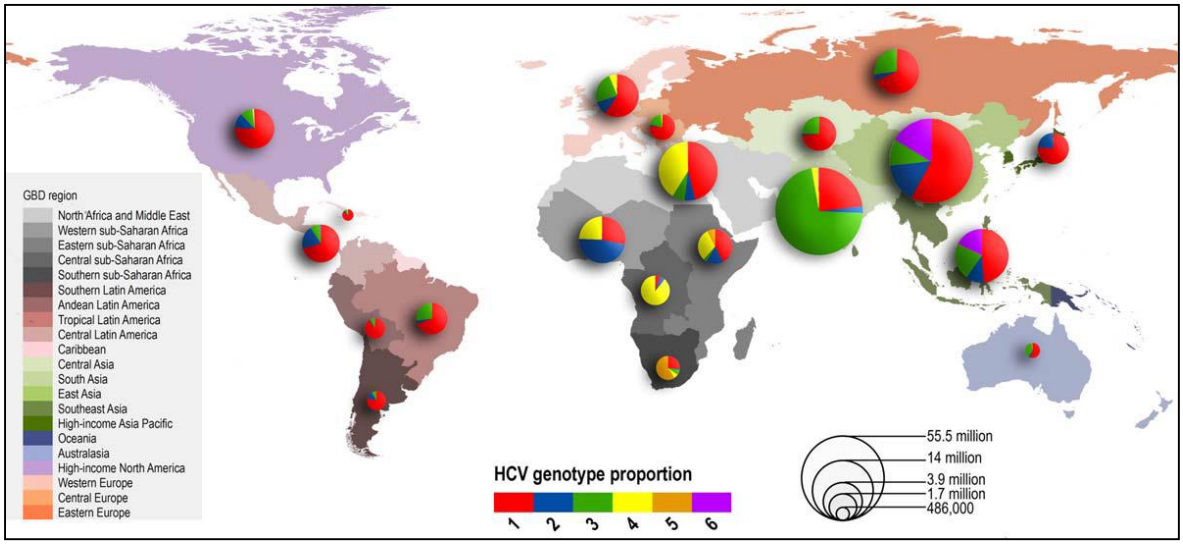


**Figure 1.7.** Relative prevalence of each HCV genotype by GBD region. The size of pie charts is proportional to the number of seroprevalent cases as estimated by Hanafiah *et al*. (142).

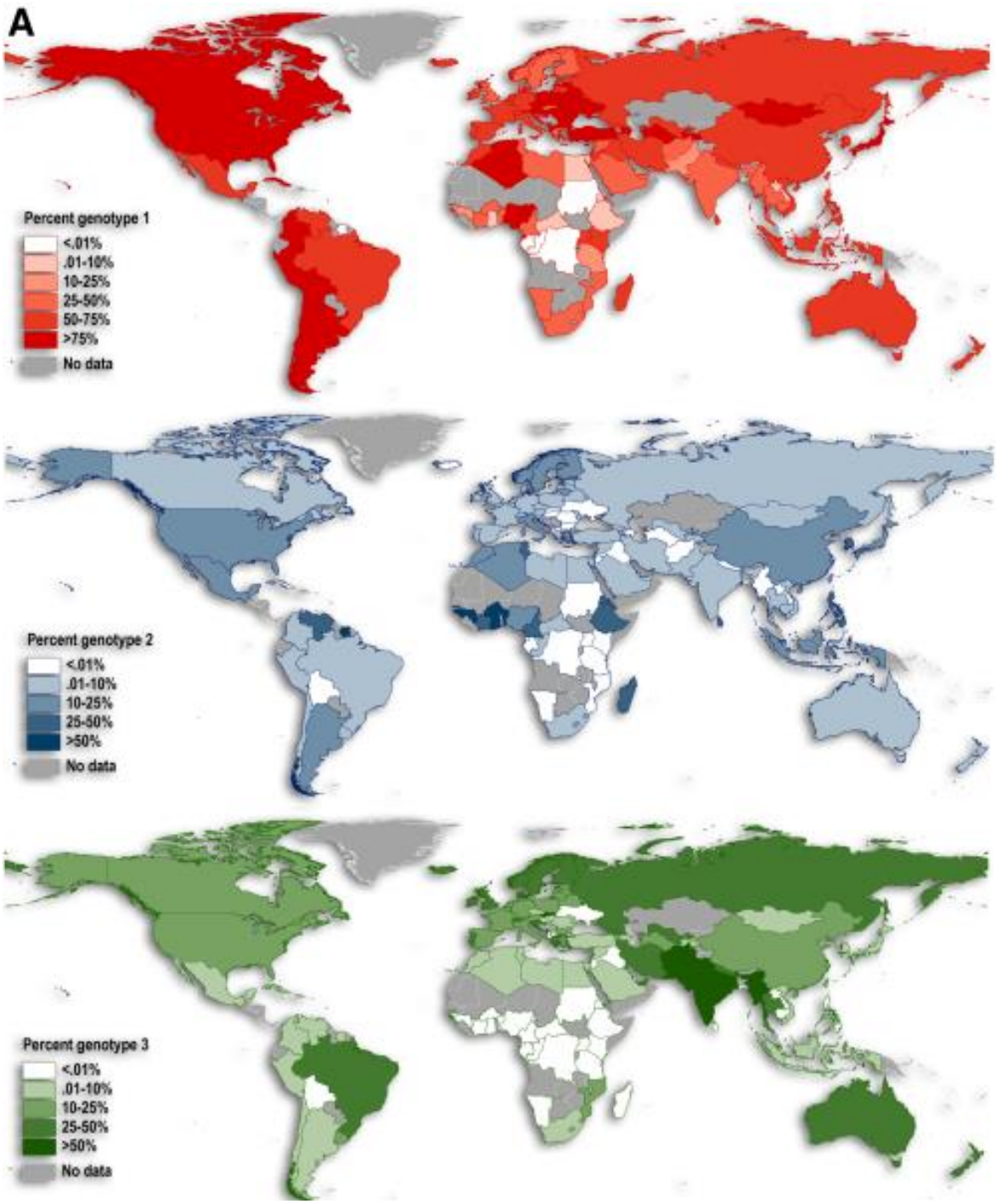


**Figure 1.8.** Relative prevalence of each genotype across all virus samples by country. Panel A: genotypes 1–3 (143).

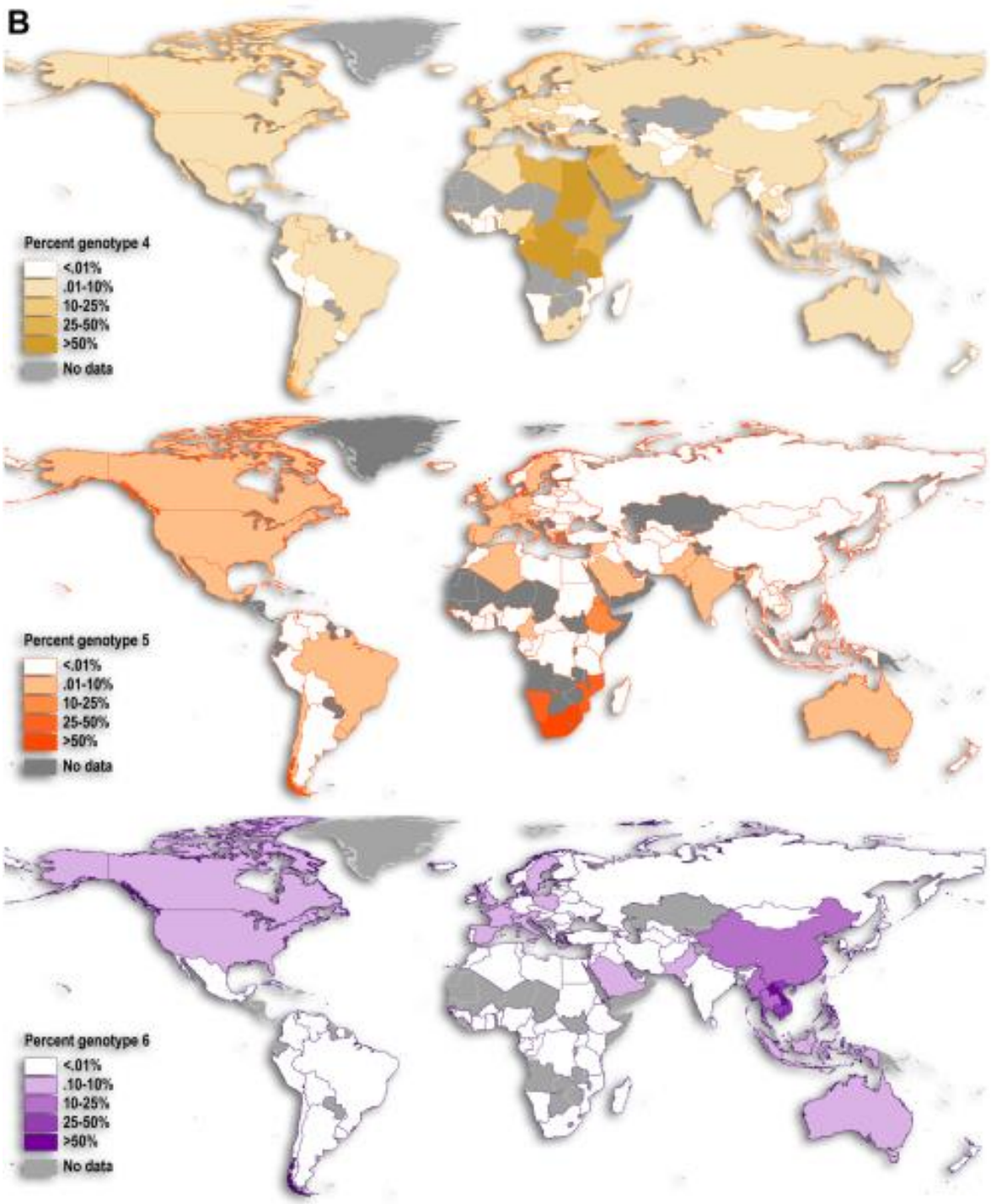


**Figure 1.8.** (Continued). Relative prevalence of each genotype across all virus samples by country. Panel B: genotypes 4–6 (143).

Another study with the objective to characterize the natural history of HCV in Portugal, including current clinical practice, annual consumption of health resources associated with treatment and follow-up of patients in the different progression

stages of the disease, using the literature review of publications between 1989 and 2013, was recently described (150). Here, it was estimated that the lower limit for the incidence of HCV infection in Portugal is at least 1 new case/100,000 persons per year and that the prevalence ranges between 1-1.5% (100,000-150,000 subjects). Furthermore, only 30% of the patients are currently diagnosed and 20% and 50% from the total number of deaths that occur by liver cirrhosis and HCC respectively are associated with HCV. Relatively to costs associated with HCV, the annual costs were about 71 million Euros of which approximately 83% (60 million Euros) are due to complications of the disease and liver transplant, with decompensated liver cirrhosis and HCC stages presenting the highest annual costs, 11,000 Euros and 17,000 Euros/patient, respectively.

### 1.15 - Transmission

The main route for HCV transmission is blood-borne including blood products or contaminated blood transfusions, needle sting in health workers, needle or syringe sharing among members of intravenous drug users (IDUs), prisoners, migrants, immigrants or refugees (151-156). Other risk factors are high-risk sexual behaviors, tattooing, shaving in a contaminated barber, reused and unsterilized dental and surgical instruments, and inaccurately prepared laboratory equipments (153, 157).

### 1.16 - Diagnosis

Diagnosis of HCV infection is based on the presence of anti-HCV antibodies using enzyme immunoassay (EIA), microparticle EIA and chemiluminescence immunoassay. The first EIAs that were developed soon after HCV discovery presented low sensitivity and evolved promptly for more sensitive and specific assays. Currently, the detection of anti-HCV antibodies against various HCV epitopes is based on the use of the third-generation EIAs in plasma or serum (158). These assays detect antibodies to recombinant antigens from structural core protein and non-structural NS3, NS4 and NS5 proteins. Moreover, the specificity and sensitivity of the third-generation immunoassays in patients with chronic liver disease were found to be >98 and >97%, respectively (159, 160). However in these cases the diagnosis is also accompanied by molecular biological tools, such as conventional PCR or real-time PCR assays as they can detect minute amounts of HCV RNA and

accurately quantify HCV RNA levels (158, 161, 162). Furthermore, the early detection of HCV in infected patients leads to decrease the number of HCV-related HCC patients. Recently biomarkers were described for this earlier detection (163). Circulating microRNAs are deregulated in liver fibrosis and HCC and most recently, serum microRNAs as potential biomarkers for early diagnosis of HCV-related HCC were described (164).

### 1.17 - Therapy

The primary goal of HCV treatment is to cure the infection. Following the European Association for the Study of the Liver (EASL) guidelines (165) until 2011, the approved treatment for chronic hepatitis C was the combination of pegylated interferon (PegIFN)-α and ribavirin for 24 or 48 weeks (162). With this regimen, patients infected with HCV genotype 1 had sustained virological response (SVR) (defined as undetectable HCV RNA 12 weeks (SVR12) or 24 weeks (SVR24) after treatment completion) rates of approximately 40% in North America and 50% in Western Europe. Higher SVR rates were achieved in patients infected with HCV genotypes 2, 3, 5, and 6 and intermediate SVR rates were achieved in those with HCV genotype 4 (166). In 2011, two first-wave, first-generation direct-acting antivirals (DAAs) drugs, telaprevir and boceprevir, were licensed for use in treatment against HCV genotype 1 infection. Both are protease inhibitors that target the HCV NS3-4A serine protease and must be administrated in combination with PegIFN-α and ribavirin. However, in HCV genotype 1 treatment-naïve patients, this triple therapy regimen achieved higher SVR rates (65% to 75%) than PegIFN-α and ribavirin dual therapy (167, 168). In 2014, three new HCV DAAs have been licensed for use as part of combination therapies for HCV infection (165). Sofosbuvir (pangenotypic nucleotide analogue inhibitor of HCV RNA-dependent RNA polymerase), simeprevir (second-wave and first-generation NS3-4A protease inhibitor active against genotypes 1 and 4), and daclatasvir (pangenotypic NS5A inhibitor). Each of these three DAAs can be used as a component of a triple combination regimen with PegIFN-α and ribavirin, yielding SVR rates of 60–100% according to the DAA used, the HCV genotype, the presence of detectable pre-existing amino acid substitutions conferring resistance to the DAA used and the severity of liver disease (165). Similar 2015 guidelines for HCV infection treatment were recommended in the USA (169),

Canada (170), and Portugal (Direcção-Geral da Saúde, Norma nº 011/2012 de 16/12/2012 actualizada a 30/04/2015).

### 1.18 - Cell Culture Systems

#### 1.18.1 - Cell Line Cultures, Cells Clones and Transfections

HCV primarily infects hepatocytes in the liver culminating in serious and progressive liver disease, as described above. However, some reports suggest that also non-hepatic reservoirs exist, including the lymphatic system, gut, and the brain (171, 172). The human hepatoma cell line (Huh)-7 seems to be the most permissive cell line for efficient RNA replication *in vitro*, as well as Huh-7 clonal derivatives (173). Initially, some studies using HCV replicons demonstrated that these have the capacity to infect the Huh-7 cells at a high level and efficiently, but this was dependent on cell density (174, 175) or cell passage number (176). Then, new studies using interferon (IFN)-*α* or a selective HCV inhibitor lead to new highly permissive Huh-7 cell derivatives, such as Huh-7.5 (177), and Huh-7-Lunet (178) among others (179, 180). However, some data that resulted from these studies were not concordant relatively to the permissiveness of individual Huh-7 clonal derivatives and the reason for this was described to be strongly related to the cell host factors that influence HCV replication (177, 179). In 2003, for the first time was reported that HCV is able to replicate in human non-liver cells as well as in murine hepatoma cells (181). Moreover, several studies also described other HCV-permissive cells in human non-liver origin, human liver-derived cells, and non-human cells (182-188). However, RNA replication is in general lower in these cells, particularly in transient replication assays, compared to Huh-7-derived cell clones. It is important to note that in all of the described systems, for HCV replication manipulated genomic HCV strains or cell cultures were addressed.

#### 1.18.2 - Primary Cells and Patient Isolates

Primary human hepatocytes (PHH) provide the closest *in vitro* model for the natural host cell of HCV. Although, PHH are of limited availability and are provided from high donor variability influencing their use in HCV research. In addition, as primary cells they rapid lose their differentiation status which also complicates tissue culture experiments. Independently of these characteristic, several groups reported

infection of cultured PHH using sera from HCV-infected patients and CD81- and LDL receptor- dependent entry of serum-derived particles or inhibition of HCV replication by interferon was also demonstrated (189-194). However, in general low-level of HCV replication and reproducibility was observed. Indeed, recently, a study which is based on *ex vivo* human adult liver slices demonstrated a productive infection using human primary isolates of HCV genotype 1b as well as HCV isolate JFH1 and genotype 1 JFH1 chimeric genomes (195). This new experimental model system allowed the detection of high viral titers (~$10^5$ focus forming units $ml^{-1}$) and validation of antiviral drugs too. Indeed, the development of more physiologically relevant infection systems is needed to provide the understanding of host–pathogen interactions in the liver.

### 1.19 - Animal Models

There is no established, small animal model that can be used to study the entire viral life cycle of HCV infection and associated immunity and pathogenesis. Chimpanzees are the only specie that is naturally susceptible to HCV infection; the other primates do not appear to be susceptible to HCV infection. Chimpanzees can be used to completely study HCV infection as they can be infected with isolates of 1-6 HCV genotypes and express innate and adaptive immune responses similar to those observed in infected humans (196, 197). While, experimentation in these large primates has yielded valuable insights, ethical considerations, limited availability, genetic heterogeneity, and cost, limit their utility. Considering all of these reasons the search for more controllable small animal models with numerous experimental approaches have been perfomed to review parts of the virus life cycle and/or aspects of viral pathogenesis (198).

In Figure 1.9 is shown an overview of described animal models used for HCV infection research, and their application in drug and vaccine development studies (198).

| |  Chimpanzees |  uPa-SCID mice |  FRG mice |  AFC8-hu HSC/hep mice |  Rosa26-Fluc mice |  Rat - immuno-tolerized |  Tree shrew |
|---|---|---|---|---|---|---|---|
| | Only complete model | Engraftment with human hepatocytes | Engraftment with human hepatocytes | Engraftment with human hepatocytes and leukocytes | Genetically humanized with HCV co-receptor molecules | Engraftment with human hepatoma cells | Non-primate mammal |
| Complete viral life cycle | Yes | Yes | Yes | No | No | Yes | Yes |
| Steps in viral life cycle | Replication[a], infection, virus production | Replication[a], infection, virus production | Infection, virus production | Infection, intrahepatic replication | Infection-intrahepatic bioluminescence expression, entry only | Infection, virus production | Infection, virus production |
| HCV genotypes | 1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a | 1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a | 1a, 2a, 3a | 1a | Not relevant | 1 | 1b, 2c, 3b, 6 |
| Culture viruses | JFH1, J6/JFH1 | JFH1, J6/JFH1 | JFH1 | None studied | JFH1- based recombinants modified to express CRE recombinase[b] | None studied | None studied |
| Viremia levels | High (4-6 $\log_{10}$ IU/mL) | High (5-7 $\log_{10}$ IU/mL) | High (5-7 $\log_{10}$ IU/mL) | None detectable | None detectable | Low | Low |
| Host responses | Innate, adaptive | Innate (gene expression) | Innate (none studied) | HCV specific cellular responses | Not relevant | Not relevant | Not relevant |
| Liver pathogenesis | Acute, chronic[c] | Not relevant | Not relevant | Hepatitis, liver fibrosis | Not relevant | Not relevant | Liver fibrosis - cirrhoses |
| **Therapeutics** | | | | | | | |
| Interferon based treatment | No effect | Antiviral effect | Antiviral effect | Not relevant | Not relevant | None studied | None studied |
| Direct acting antiviral (DAA) agents | Protease/ polymerase | Entry, protease, NS5A, polymerase | None studied | Not relevant | Not relevant | None studied | None studied |
| Host factor directed drugs | miR-122 antagonist | Cyclophilin inhibitor, and others | Cyclophilin inhibitor | Not relevant | Not relevant | None studied | None studied |
| **Passive immuno prophylaxis** | | | | | | | |
| Polyclonal human anti-HCV | Delayed viremia | Cross-genotype protection | None studied | None studied | None studied | None studied | None studied |
| Monoclonal human anti-HCV | None studied | Cross-genotype protection | None studied | None studied | Protective (anti-E2) | None studied | None studied |
| HCV receptor blocking antibodies | None studied | Protective (anti-CD81, anti-SR-BI) | None studied | None studied | Protective (anti-CD81) | None studied | None studied |
| **Vaccines** | | | | | | | |
| Therapeutic | Only available model | Not relevant | Not relevant | Not relevant | Not relevant | Not relevant | Not relevant |
| Prophylactic | Only available challenge model | Not relevant | Not relevant | Not relevant | Neutralizing antibodies | Not relevant | Not relevant |

**Figure 1.9.** Proposed animal models in HCV research (198). Specific characteristics on utility and applications are indicated for each model. For all small animal models some areas of research are not currently feasible, which are indicated as "not relevant". [a]Tested by intrahepatic transfection with RNA transcripts from full-length HCV clones. [b]Activates a cellular reporter permitting bioluminescence imaging. [c]Chimpanzees are not necessarily good models of chronic liver disease and HCV-associated HCC.

Following the information provided in Figure 1.9 and all reviewed information by Jens Bukh in 2012 (198), Chimpanzees currently remain the only model available for HCV research including studies of infection and disease progression as well as for prophylactic and therapeutic vaccines. Moreover, these animals are also

important in microRNA studies as shown in a study referring the antagonist of miR-122 (199). Certainly, the creation of mice with human hematopoietic and liver cells allowed researchers to reproduce HCV induced liver fibrosis in mice (200). This is one of the limitations of chimpanzees use for studies of HCV infection as they are not good models of chronic liver disease and HCV-associated HCC (198).

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# CHAPTER 2

# STUDY HYPOTHESIS AND AIMS

## 2.1 - Study Hypothesis

During an investigation addressing the *Flaviviridae* family in the search for significant natural virus's reservoirs of animal diseases we have tested body fluids and liver homogenates samples from domestic and wild European rabbit (*O. cuniculus*) and hare (*L. europaeus*) for Bovine Virus Diarrhea Virus (BVDV) antigen detection using a commercial blocking Enzyme-Linked Immunosorbent Assay (ELISA) (SerelisaTM BVD p80 Ag Mono Indirect, Symbiotics, Lyon - France) and all tested positive. However, when we tested serum and body fluid samples of those animals in the study using an antibody ELISA BVD p80 Ab Mono Blocking detection kit) that detects specific antibodies to a protein common to all strains of bovine viral diarrhea/mucosal disease (BVD/MD) and border disease (BD) virus (p80/125 non-structural protein) they all tested negative. Efforts to detect significant *Pestivirus* proteins were unsuccessful. These observations led us to hypothesize that potential protein cross reactivity within members of the *Flaviviridae* family could explain the results in the preliminary work described and thus, this study was initiated.

## 2.2 - Aims of the Study

In general, this work aimed to contribute in the knowledge of the role that European rabbit (*O. cuniculus*) and European hare (*L. europaeus*) may present as natural reservoirs or precursors of viruses that have a significant impact in diseases of animals and humans.

In order to reach this aim, samples from Portuguese populations of European rabbits (domestic and wild) and hare were processed following four proposed objectives:

1. Detection of HCV specific regions by PCR and RT-PCR, using specific primers and sequencing.
2. Identification and characterization of specific HCV proteins.
3. Test the biological activities of the identified and characterized proteins in cell cultures.
4. Data and phylogenetic analysis.

# CHAPTER 3

# DETECTION AND CHARACTERIZATION OF HCV HOMOLOGOUS FRAGMENTS IN THE EUROPEAN RABBIT AND EUROPEAN HARE

## 3.1 - HCV Homolog Fragments, Cell-Lines and Applications Thereof

## A B S T R A C T

**"HCV HOMOLOG FRAGMENTS, CELL-LINES AND APPLICATIONS THEREOF"**

The present subject matter relates to fragments homologous to HCV structural and non-structural (NS) proteins present in the European rabbit (Oryctolagus cuniculus) and/or hare (Lepus europaeus) genomes which infect and autonomously replicate in permissive bovine cell cultures, Mardin-Darby Bovine Kidney (MDBK), epithelial cell line and primary bovine testicle (BT) cells, respectively. An aspect disclosed relates to isolated fragments for an in vitro model of HCV replication in bovine cell cultures and cell cultures for an in vitro model of HCV replication, having at least one of the isolated nucleotide or peptide sequences described. The cell cultures for use in medicine, preferably to the use of cell cultures as in vitro models for HCV replication.

# D E S C R I P T I O N

## "HCV HOMOLOG FRAGMENTS, CELL-LINES AND APPLICATIONS THEREOF"

### Technical field

The present disclosure relates to fragments homologous to HCV structural and nonstructural (NS) proteins present in particular in the European rabbit (Oryctolagus cuniculus) and/or hare (Lepus europaeus) genomes that infect and autonomously replicate in permissive cell cultures.
The disclosure also includes the use of HCV homolog synthetic nucleotides or peptides that infect and autonomously replicate in permissive cell cultures, and Human HCV isolates that infect and replicate in permissive cell cultures. The present disclosure provides methods and tools that can contribute in identifying novel antivirals, vaccine production, new diagnostic tools as well as a better understanding of the HCV life cycle, its origin and diversity, the pathogenesis mechanisms of this virus using these cells as models. The present disclosure relates to permissive cell cultures in particular bovine cell cultures.

### Background

Endogenous retroviruses, non-retroviral RNA viruses and DNA viruses have been found in the mammalian genomes. Among these, Human Retrovirus 5 and endogenous lentivirus were found to be present in European rabbits. The European rabbit, native to the Iberian Peninsula, is the single recognized progenitor of domestic rabbits. Rabbits have many hereditary diseases common to humans like aortic arteriosclerosis, hypertension, hypertrophic cardiomyopathy, osteoporosis, making them a valuable model in both biomedical and fundamental research.

Hepatitis C virus (HCV) origin remains unclear since its discovery largely because a closely related animal homolog virus has not been identified, however a canine homolog of HCV has been recently described. Unlike most RNA viruses which usually cause acute diseases, HCV establishes life-long, persistent, intrahepatic infections in the majority of the infected individuals, frequently leading to the development of cirrhosis and hepatocellular carcinoma and affects about 170 million people worldwide. HCV is a plus positive single-stranded RNA virus, so called because upon infection their RNA can be directly translated into protein by host machinery, with envelope that belongs to the Flaviviridæ family. Its genome, of ~9.6 kb, contains one large open reading frame that encodes one large polyprotein that is processed by viral and cellular proteinases to produce the virion structural proteins (core and envelope glycoprotein's E1 and E2) as well as NS proteins (P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B) that form the replication

complex. NS3 to NS5B proteins are necessary and sufficient to establish membrane-bound replication complexes that catalyze RNA replication and NS5B protein codes RNA-dependent RNA polymerase (RdRp), because it has a Gly-Asp-Asp (GDD) motif which serves to replicate the HCV-RNA genome.

The current treatment for chronic HCV-infected patients is the combination therapy with pegylated alpha-interferon and ribavirin but is not always efficient and the mechanism of this treatment is poorly understood, known with a small efficacy and with severe adverse side effects. However several approaches are currently in development targeting virus replication cycle using protease inhibitors, non-nucleoside inhibitors that target and directly bind to the RNA dependent RNA polymerase.

Despite the important role of HCV in human health there are still several unknowns to be clarified. Achievements have been obtained up to date about the virus, but the details of the life cycle are largely unknown. In 2005 a robust *in vitro* replication model of HCV was developed with combined efforts of many scientists. Numerous efforts have been conducted in the past to establish a cell culture system so that the various pathways involved in HCV induced pathogenesis would be studied and preventive strategies against this pathogen would become available (document WO 2010/017818). Investigation has however some drawbacks, such as, the use of genetic manipulations to modify the virus in order to achieve *in vitro* tools for research.

The existing animal models for HCV pathogenesis studies are also limited, remaining the chimpanzee as the best animal model for studying the biology of HCV. However the development of smaller animal models may be continued, that can provide a large number of animals to be employed with statistical significant data making this a major advantage.

The development of antiviral drugs and the molecular studies of HCV have been hampered by a lack of a reliable cell culture system like hepatoma cell lines, African green monkey Vero cells, mosquito cells allowing a persistent in vitro virus replication and viral adaptation to the culture (V. Lohmann et al., Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110-113 (1999); B. D. Lindenbach et al., Complete replication of hepatitis C virus in cell culture. Science 309, 623-626 (2005); J. Guo, R. Yan, G. Xu, W. Li, C. Zheng, Construction of the Vero cell culture system that can produce infectious HCV particles. Mol. Biol. Rep. 36, 111-120 (2009); R. Germi et al., Mosquito cells bind and replicate hepatitis C virus. J. Med. Virol. 64, 6-12 (2001)).

One of the advantages of the present disclosure is being a fast and simple *in vitro* model that is reproducible, reliable and faster.

**Summary**

Before describing the disclosure in detail, it is to be understood that the terminology used herein is for the purpose of describing particular embodiments of the present subject matter only, and is not intended to be limiting.

*Definitions*

Although a number of materials and methods similar or equivalent to those described herein can be used in the practice of the present disclosure the preferred ones are described herein.

The term “fragments” are nucleotide sequences and/or peptide sequences, more or less longer in length.

In the present disclosure samples, e.g., liver homogenates from 6 wild and 6 domestic rabbits and 1 hare, 5 serum and WBC samples of domestic rabbits and 7 body fluids of the 6 wild rabbits and hare were used.

In this disclosure total RNA was extracted from 10% (w/v) liver homogenates with TRI® Reagent LS (preferably Sigma-Aldrich, Steinheim, Germany). From the RNA extracts cDNA was synthesized using random hexamers (preferably Amersham, New Jersey, USA). Genomic DNA was extracted from the same samples as RNA using preferably QIAamp® DNA blood kit (preferably Qiagen, Hilden, Germany) according to the manufacturer instructions.

After, in this disclosure using cDNA and DNA, RT-PCR and PCR were performed targeting the specific HCV structural and NS proteins, core, envelope glycoprotein’s E1/E2, NS5A/B and NS5B, in a thermocycler C 1,000 (preferably Bio-Rad, California, USA). PCR cycling conditions were performed as previously described (H. Okamoto et al., Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J. Gen. Virol. 73, 673-679 (1992); J. Verbeeck et al., Investigating the origin and spread of hepatitis C virus genotype 5a. J. Virol. 80, 4220-4226 (2006); N. Enomoto, A. Takada, T. Nakao, T. Date, There are two major types of hepatitis C virus in Japan. Biochem. Biophys. Res. Commun. 170, 1021-1025 (1990); K. Sandres-Saune et al., Determining hepatitis C genotype by analyzing the sequence of the NS5b region. J. Virol. Methods 109, 187-193 (2003); T. Nakao, N. Enomoto, N. Takada, A. Takada, T. Date, Typing of hepatitis C virus genomes by restriction fragment length polymorphism. J. Gen. Virol. 72, 2105-2112 (1991)) with the following primers:

- Core 256 (SEQ ID NO: 53): 5`-CGCGCGACTAGGAAGACTTC-3`
- Core 186 (SEQ ID NO: 54): 5`-ATGTACCCCATGAGGTCGGC-3`
- Core 104 (SEQ ID NO: 55): 5`-AGGAAGACTTCCGAGCGGTC-3`
- Core 134 (SEQ ID NO: 56): 5`-CCAAGAGGGACGGGAACCTC-3`
- E1/E2 HVR1F (SEQ ID NO: 57): 5`-TGCTGGGTCCARRTYACCCC-3`
- E1/E2 HVR1R (SEQ ID NO: 58): 5`-GCTGTCATTACAGTTAAGGGCA-3`
- NS5A/B S (SEQ ID NO: 59): 5`-TGGGGATCCCGTATGATACCCGCTGCTTTGA-3`
- NS5A/B AS (SEQ ID NO: 60): 5`-GGCGGAATTCCTGGTCATAGCCTCCGTGAA-3`
- NS5A/B S` (SEQ ID NO: 61): 5`-TGCGGTTATTGCCGTTGTCGCGCCAGCGG -3`
- NS5A/B AS` (SEQ ID NO: 62): 5`-GGCAGAATACCTAGTCATGGCCTCTGTGAA-3`
- NS5B Pr3 (SEQ ID NO: 63): 5`-TATGAYACCCGCTGYTTTGACTC-3`
- NS5B Pr4 (SEQ ID NO: 64): 5`-CNGARTAYCTVGTCATAGCCTC-3`.

The amplicons were directly sequenced after being purified using a Qiagen kit (preferably Qiagen, QIAquick® Gel Extraction Kit, Crawley, UK) using the same primers.

An aspect of disclosed subject matter relates to Isolated fragments for an in vitro model of HCV replication in cell lines, said fragments having the sequence with least 95% of homology of at least one of SEQ ID No. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 36, 37, 38, 39, 40, 41, 42 , 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 with the capability to replicate autonomously when inoculated on a cell line permissible to the said sequences.

In embodiments of the disclosure said fragments may be from rabbit or hare.

Others embodiments of the disclosure, the said cell lines permissible can be bovine cell cultures, preferably MDBK or BT.

A further aspect of the disclosure relates to the cell cultures for an in vitro model of HCV replication, having at least one of the isolated fragment sequences having at least one of the isolated nucleotide or peptide sequences with at least 95% of homology of at least one of SEQ ID No. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42 , 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 with the capability to replicate autonomously when inoculated on the cells permissible to the said sequences.

In embodiments of the disclosure, the permissible cell cultures can be bovine cell cultures, preferably MDBK or BT.

Others embodiments of the disclosure, the cell cultures for use in medicine.

An aspect of disclosed subject matter relates to the use of cell cultures as in vitro models for HCV replication.

A further aspect of the disclosure relates to a method for evaluating the viability or metabolism in cell cultures for develop diagnostic/therapeutic methods for HCV after contact with a test substance, cell or protein which comprises the following steps: contacting a test substance, cell or protein with cell cultures for an in vitro model of HCV replication cells and analyze the viability or metabolism of the cell culture.

In embodiments of the disclosure, the test substances are anti-nucleases, protease inhibitors, cell receptors blocking, among others.

An aspect of disclosed subject matter relates to a kit for evaluating the viability or metabolism in cell cultures for develop diagnostic/therapeutic methods for HCV after contact with a test substance, comprising cell cultures for an in vitro model of HCV replication cells described above.

In one embodiments of the disclosure, the disclosure will be used as a model to unravel the origin of the HCV and/or the HCV diversity and/or the permissive mechanisms for HCV replication.

In embodiments of the disclosure, the fragments and/or cell lines and/or kit can be used as methods and tools to study the active/ repressive mechanisms for HCV replication and/or to detect HCV infectivity markers and/or detect HCV immunodominant markers.

An aspect of disclosed subject matter relates to a system to develop novel early diagnostic methods for HCV and/or cell cycle and mechanisms involved and/or to study HCV pathogenesis mechanisms such as: virus receptors, oxidative-stress mechanisms, lipid vesicles, apoptosis, tumor markers and others and/or to study HCV genes and proteins functions and/or to study HCV scape mechanisms to the immune system and/or to identify efficient antiviral drugs for HCV such as: anti-nucleases, protease inhibitors, cell receptors blocking, and/or others.

In embodiments of the disclosure, the fragments and/or cell lines and/or kit can be used for HCV propagation and/or titration.

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In embodiments of the disclosure, the fragments and/or cell lines and/or kit can be used be used as a model to be used in vaccines development against viruses such as: HCV.

**General Description**

The present subject matter provides methods and tools that can contribute in identifying novel antivirals, vaccine production, new diagnostic tools as well as a better understanding of HCV origin and life cycle, diversity and pathogenesis mechanisms using the rabbit, hare and bovine cells as models.

The present disclosure also relates to fragments homologous to HCV structural and non-structural (NS) proteins present in the European rabbit (Oryctolagus cuniculus) and/or hare (Lepus europaeus) genomes which infect and autonomously replicate in permissive bovine cell cultures, Mardin-Darby Bovine Kidney (MDBK) (Madin SH, Darby NB Jr.. Established kidney cell lines of normal adult bovine and ovine origin. Proc. Soc. Exp. Biol. Med. 98: 574-576, (1958)) epithelial cell line and primary bovine testicle (BT) cells, respectively. The HCV genomic homolog fragments were demonstrated by RT-PCR, PCR, mass spectrometry and replication in cell cultures by immunofluorescence assay (IFA) and immunogold electron microscopy (IEM) using specific monoclonal Abs (Mabs) for HCV NS3, NS4A and NS5 proteins.

The subject matter also includes rabbit and hare genomic DNA RNAse treated that infect and replicate in permissive bovine cell cultures. HCV RNA titers were quantified using Real Time-PCR and replication in bovine cell line described above by immunofluorescence assay (IFA) using specific monoclonal Abs (Mabs) for HCV NS3 and NS5 proteins, and immunogold electron microscopy (IEM) using specific monoclonal Abs (Mabs) for HCV-E2 proteins.

The present disclosure also includes the use of HCV homolog synthetic nucleotides or peptides that infect and autonomously replicate in bovine cell cultures. HCV RNA titers were quantified using Real Time-PCR.

The deisclosure also includes Human HCV isolates that infect and replicate in permissive bovine cell cultures. HCV RNA titers were quantified using Real Time-PCR and replication in bovine cell line described above by immunofluorescence assay (IFA) using specific monoclonal Abs (Mabs) for HCV NS3, NS4A and NS5 proteins.

Results obtained in this disclosure led us to conclude that bovine cells are permissive cells to HCV in particular MDBK and/or BT cells and can unravel functions to know of the virus pathogenesis, its prevention and control, leading to novel research approaches. In another embodiment both animals and bovine cell models can be used in research in search of intrinsic

permissive and non-permissive factors for the virus replication, discovery of new therapies, new early diagnostic tools, for preventive options against HCV infection.

**Description of the figures**

The following figures provide preferred embodiments for illustrating the description and should not be seen as limiting the scope of disclosure.

**Figure 1. Detection of HCV proteins by immunofluorescence in MDBK and BT cells.** The cells infected with liver homogenates from domestic (DR), wild (WR) rabbits and a hare (H) were analyzed 14 days post infection. MDBK and BT cells were incubated with MAbs specific for NS3, NS4A and NS5 proteins. Infected cells demonstrated fluorescence (2). Negative controls (NC) for each cell culture and each Mab were also performed. No fluorescence was detected (1). Scale bars: (NS3 and NS4A MAbs for MDBK cells), 20 µm; (NS5 MAbs for MDBK cells, and all MAbs for BT cells), 50 µm.

**Figure 2. Immunogold TEM detection of HCV proteins in MDBK cells.** The cells infected with liver homogenates from domestic (DR), wild (WR) rabbits and a hare (H), were analyzed 14 days post infection. The cells were incubated with MAbs specific for NS3, NS4A and NS5 proteins. Negative controls (NC) for each Mab were also performed and no immunogold particles were detected (1). The immunogold particles were 10 nm in diameter (2). Scale bars: 100 nm.

**Figure 3a, 3b and 3c. MALDI-TOF/TOF-MS/MS spectra of the peptide sequences identified with HCV homolog fragments on liver samples.** Domestic rabbits (1-3), wild rabbits (4-6) and hare (7-9). Da – Dalton; C. I. – confidence interval; TWAQPGYPWPLYGNEGMGWAGWLLSPR (1), VASSTQSLVSWLSQGPSQK (2), AAIRSLTQR (3), AKAPPPSWDAMWKCLAR (4), IVGPKMCSNVWNNR (5), SASLRQK (6), LGKEVLLGPADDYR (7), NGSMRLAGPR (8), DVRSHTSK (9). For more detailed information refer to Table 2.

**Figure 4. Immunogold TEM detection of HCV E2 protein in MDBK cells.** The cells infected with liver homogenates (LH) from domestic rabbit (DR) and a hare (H), were analyzed 28 days post infection (P4). The cells infected with DNA extracted from liver homogenates from domestic rabbit (DR) and a hare (H) (DNA) were analyzed 35 and 49 days post infection (P5 and P7) respectively. The cells were incubated with MAbs specific for E2 protein. Negative controls (NC)

were also performed and no immunogold particles were detected (1). The immunogold particles were 10 nm in diameter (2). Scale bars: 200 nm.

**Figure 5. Detection of HCV specific proteins by immunofluorescence in MDBK cells infected with HCV-1a.** The cells infected with HCV genotype -1a were analyzed at several time points and incubated with MAbs specific for NS3 and NS5 proteins. Infected cells demonstrated fluorescence (2). Negative controls (NC) for each cell culture and each Mab were also performed. No fluorescence was detected (1). Scale bars: 10 µm.

**Figure 6.** Antiviral effect of Interferon-α2b (500IU/ml), Ribavirin (20µM), Danoprevir (100nM) and combination of them, on HCV-1a replication in MDBK cells. Data are expressed as $Log_{10}$ (IU/ml).

**Detailed description of the disclosure**

The present disclosure provides fragments homologous to HCV structural and nonstructural (NS) proteins present in the European rabbit (Oryctolagus cuniculus) and/or hare (Lepus europaeus) genomes that infect and autonomously replicate in permissive bovine cell cultures.

In an embodiment, the fragments homologous to HCV were carried out by RT-PCR and PCR using extracted RNA and DNA respectively from liver homogenates (6 wild and 6 domestic rabbits and 1 hare) and sequenced all amplified products obtained with these methods. Core, E1/E2, NS5A/B and NS5B HCV genomic proteins were successfully amplified from all samples (data not shown), when specific primers were used in both PCRs as described above. RT-PCR from NS5A/B HCV proteins was also performed in white blood cells and serum samples from 5 domestic rabbits and in body fluids from 6 wild rabbits and one hare and amplified fragments were only detected on white blood cells. Sequencing of RT-PCR and PCR amplified regions was performed. Alignments and tblastn searches were conducted as a query in the HCV database and the retrieved fragment sequences from core, E1/E2, NS5A/B and NS5B HCV specific proteins were identified (Table 1).

Table 1.

| SEQ ID No. | HCV regio | Primers | Identified nucleotides | Acession no. | Genotype | Position | E-value | % Identities |
|---|---|---|---|---|---|---|---|---|
| SEQ ID No. 1 | Core | 104/134 | GTGACCGCTCGGAAGTCTTCC[a] | GU441256 | 1b | 304-284* | 0.005 | 100 |
| SEQ ID No. 2 | Core | 104 | CGAGGTTCCCGTCCCTCTTGG | U10230 | 2a | 301-321 | 0.33 | 100 |
| SEQ ID No. 3 | Core | 134 | CGACCGCTCGGAAGTCTTCCTA | HM049503 | 2a | 508-487 | 0.004 | 100 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| SEQ ID No. 4 | E1/E2 | HVR1F/R | TTTGGAAAAGGCCAGGGAA | AM885177 | 1a | 232-214* | 0.37 | 94 |
| SEQ ID No. 5 | E1/E2 | HVR1R | TTTTGTTTCCGAAGCCAATACAT | AY767496 | 3a | 133-158 | 0.12 | 84 |
| SEQ ID No. 6 | E1 | HVR1F | CCA[b] CCACCAAAACCGTCCCTGGCCT | AM885166 | 1a | 203-232 | 2.4 | 80 |
| SEQ ID No. 7 | NS5B | AS/S | TTTCCAAA GCTGGGGACTTGTGCCTTCAGG | EF116149 | 6a | 235-204* | 0.014 | 81 |
| SEQ ID No. 8 | NS5B | S | GGACATGTGG AGCCTCCGTGAAAGCTTG | HM009084 | 1b | 303-286* | 1.1 | 94 |
| SEQ ID No. 9 | NS5B | S | CAAGCTTTCACGGAGGCTATGA | AY685047 | 4p | 366-401* | 4e-08 | 100 |
| SEQ ID No. 10 | NS5B | AS | CCAGGAATTCCGCC AATCAAAGCAGCGGGTATCATA | AY743071 | 4p | 33-1* | 8e-07 | 100 |
| SEQ ID No. 11 | NS5B | S` | CGGGATCCCCA TCTTCACGGAGGCTATGACTAG | GU589872 | 1 | 348-379 | 2e-04 | 93 |
| SEQ ID No. 12 | NS5B | AS` | GTATTCTGCC GATCCCGTATGATACCCGCTGC | DQ508484 | 1b | 5-31 | 6e-06 | 100 |
| SEQ ID No. 13 | NS5B | AS` | TTTGA TCAAAGCAGCGGGTATCATACG | DQ345619 | 1b | 32-2* | 6e-05 | 100 |
| SEQ ID No. 14 | NS5B | Pr3/Pr4 | GGATCCCCA GCTGGGGACTTGTGCCTTCAGG | EF116149 | 6a | 235-204* | 0.014 | 81 |
| SEQ ID No. 15 | NS5B | Pr3/4 | GGACATGTGG AGCTTTCACGGAGGCTATGACC | DQ238690 | 2b | 66-98 | 8e-09 | 100 |
| SEQ ID No. 16 | NS5B | Pr3/4 | AGGTACTCAGC[c] AGCTTTCACGGAGGCTATGACC | DQ663603 | 3a | 327-360 | 4e-07 | 100 |
| SEQ ID No. 17 | NS5B | Pr4 | AGATATTCAGCA TATGATACCCGCTTGCTTTGA | X88622 | 1a | 14-29 | 1.1 | 100 |
| SEQ ID No. 18 | NS5B | Pr4 | GTAGAGTCGAAGCAACGGGTAA CATA | EU255955 | 1a | 8190-8165 | 0.023 | 96 |

Nucleotide homology between the studied samples and HCV sequences deposited at the site (http://hcv.lanl.gov/content/sequence/BASIC_BLAST/basic_blast.html) are labeled in bold and underlined. * Reverse complement.

In the present disclosure proteins were extracted from 10% (w/v) liver homogenates following the Alliance for Cellular Signaling (preferably AFCS) protocol. Protein concentration was determined in a Qubit® Fluorometer (preferably Invitrogen, Carlsbad, USA) according to the manufacturer instructions. After, liver homogenate proteins from rabbit and hare were separated by SDS-PAGE and stained with preferably Coomassie Blue. The visible protein bands that were selected on the basis of their molecular weight and that could harbor proteins matching the core, envelope glycoprotein's E1 and E2, serine protease NS3, NS4B, NS5A and NS5B HCV genomic proteins, were excised from the gel, and analyzed by MALDI-TOF/TOF-MS/MS as described by Pinho *et al.* For each digested sample, the MS and MS/MS spectra were combined for the MASCOT database search. With the matched MS/MS spectra of the European rabbit and *Lepus europaeus* to HCV sequences deposited at Swiss-Prot/UniProt protein database a total of 34 peptide sequences were identified, such as core, envelope glycoprotein's E1 and E2, protease NS2–3, serine protease NS3, NS5A and RdRp-NS5B HCV specific proteins (Table 2).

Table 2.

| SEQ ID No. | Animal | Accession no.† | Protein name | Best peptide sequence | Genome position | Match error (Da) | Total ion score C I % | E-value |
|---|---|---|---|---|---|---|---|---|

| | | (genotype) | | | (aa) | | | |
|---|---|---|---|---|---|---|---|---|
| SEQ ID No. 19 | DR | POLG_HCVJA (1b) | Core | STNPKPQR | 2-9 | -0.02 | 99 | 0.39 |
| SEQ ID No. 20 | DR | POLG_HCVJA (1b) | Core | SQPRGRR | 56-62 | -0.02 | 99 | 0.23 |
| SEQ ID No. 21 | DR | POLG_HCVJA (1b) | Core | TWAQPGYPWPLYGN EGMGWAGWLLSPR | 75-101 | 0.07 | 99 | 0.3 |
| SEQ ID No. 22 | DR | POLG_HCVJA (1b) | E1 | NSSIPTTTIRR | 250-260 | -0.04 | 99 | 0.022 |
| SEQ ID No. 23 | DR | POLG_HCVJA (1b) | E2 | VASSTQSLVSWLSQ GPSQK | 392-410 | -0.07 | 98 | 0.046 |
| SEQ ID No. 24 | DR | POLG_HCVVA (2K) | E2 | SIEEFR | 461-466 | 0.03 | 71 | 0.58 |
| SEQ ID No. 25 | DR | POLG_HCVBK (1b) | NS2-3 | KVAGGHYVQMAFMK ** | 927-940 | -0.04 | 95 | 0.047 |
| SEQ ID No. 26 | DR | POLG_HCVJA (1b) | NS3 | GPITQMYTNVDQDLV GWPAPPGAR | 1095-1118 | 0.06 | 81 | 0.6 |
| SEQ ID No. 27 | DR | POLG_HCVJ1 (1b) | NS3 | AVDFIPVESLETTMR | 1192-1206 | -0.07 | 87 | 0.049 |
| SEQ ID No. 28 | DR | POLG_HCVJA (1b) | NS5A | DVWDWICTVLSDFKT WLQSKLLPR* | 1979-2002 | 0.09 | 81 | 0.013 |
| SEQ ID No. 29 | DR | POLG_HCVT5 (6b) | NS5A | IPGIPFISCQAGYR* | 2008-2021 | -0.01 | 89 | 0.15 |
| SEQ ID No. 30 | DR | POLG_HCVK3 (3a) | NS5A | NGSMRLAGPR** | 2047-2056 | -0.02 | 74 | 0.92 |
| SEQ ID No. 31 | DR | POLG_HCVSA (5a) | NS5A | GSPPSLASSSASQLS APSLK | 2194-2213 | -0.08 | 90 | 0.087 |
| SEQ ID No. 32 | DR | POLG_HCVJA (1b) | NS5B | VEFLVNTWK | 2620-2628 | 0.01 | 81 | 0.15 |
| SEQ ID No. 33 | DR | POLG_HCVSA (5a) | NS5B | AAIRSLTQR | 2674-2682 | -0.02 | 99 | 0.1 |
| SEQ ID No. 34 | DR | POLG_HCVJA (1b) | NS5B | AFTEAMTR | 2757-2764 | -0.01 | 81 | 1.1 |
| SEQ ID No. 35 | WR | POLG_HCVNZ (3a) | Core | SQPRGRR | 56-62 | 0.04 | 22 | 1.1 |
| SEQ ID No. 36 | WR | POLG_HCV6A (6a) | NS3 | CDELAGKLKSLGLNA VAFYR* | 1405-1424 | -0,04 | 87 | 0.15 |
| SEQ ID No. 37 | WR | POLG_HCVJ8 (2b) | NS3 | GRLGVYR | 1498-1504 | -0.04 | 57 | 0.05 |
| SEQ ID No. 38 | WR | POLG_HCVJF (2a) | NS3 | AKAPPPSWDAMWKC LAR* | 1601-1617 | 0.04 | 93 | 0.016 |
| SEQ ID No. 39 | WR | POLG_HCVVO (6K) | NS5A | NGSMRISGSR | 2043-2052 | 0.04 | 22 | 0.05 |
| SEQ ID No. 40 | WR | POLG_HCVVN (6d) | NS5A | IVGPKMCSNVWNNR* | 2044-2057 | 0.01 | 87 | 0.2 |
| SEQ ID No. 41 | WR | POLG_HCVCO (1b) | NS5A | VGDFHYVTGMTTDN VK** | 2096-2111 | 0.06 | 74 | 0.5 |
| SEQ ID No. 42 | WR | POLG_HCVCO (1b) | NS5A | GSPPSLASSSASQLS APSLK | 2193-2212 | -0.08 | 74 | 0.53 |
| SEQ ID No. 43 | WR | POLG_HCVJ6 | NS5A | SDLEPSIPSEYMLPK | 2264-2280 | -0.05 | 22 | 0.1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | (2a) | | KR | | | | |
| SEQ ID No. 44 | WR | POLG_HCV6A (6a) | NS5B | SASLRQK | 2472-2478 | 0.00 | 87 | 0.13 |
| SEQ ID No. 45 | WR | POLG_HCVJ8 (2b) | NS5B | LLTVEEACALTPPHS AK* | 2524-2540 | -0,05 | 57 | 0.15 |
| SEQ ID No. 46 | WR | POLG_HCV6A (6a) | NS5B | MALYDVTR** | 2601-2608 | 0.01 | 87 | 0.2 |
| SEQ ID No. 47 | Hare | POLG_HCVJP (2b) | Core | GSRPTWGPSDPRHR | 102-115 | 0.04 | 53 | 0.13 |
| SEQ ID No. 48 | Hare | POLG_HCVJP (2b) | E2 | LWHYPCTVNFTIFKV R* | 619-634 | -0.04 | 53 | 0.65 |
| SEQ ID No. 49 | Hare | POLG_HCVJK (3K) | NS2-3 | LGKEVLLGPADDYR | 1011-1024 | -0.07 | 81 | 0.067 |
| SEQ ID No. 50 | Hare | POLG_HCVK3 (3a) | NS5A | NGSMRLAGPR** | 2047-2056 | -0.05 | 67 | 0.92 |
| SEQ ID No. 51 | Hare | POLG_HCVJP (2b) | NS5B | AASKVSAR | 2516-2523 | 0.00 | 53 | 0.65 |
| SEQ ID No. 52 | Hare | POLG_HCVT5 (6b) | NS5B | DVRSHTSK | 2535-2542 | 0.04 | 82 | 0.09 |

DR – Domestic rabbit *(Oryctolagus cuniculus)*, WR – Wild rabbit *(Oryctolagus cuniculus.)*, Hare (*Lepus europaeus*), † – SwissProt accession number,*Modification - Carbamidomethyl (C), ** Modification - Oxidation (M).

With MS/MS spectra from domestic rabbit we were able to identify 16 peptide sequences that match core, envelope glycoprotein's E1 and E2, protease NS2–3, serine protease NS3, NS5A and RdRp-NS5B HCV specific proteins, 12 peptide sequences that match to the core, serine protease NS3, NS5A and RdRp-NS5B HCV specific proteins were identified in wild rabbits, and from hare we were able to identify 6 peptide sequences that match core, envelope glycoprotein E2, protease NS2–3, NS5A and RdRp-NS5B HCV specific proteins (Figure 3).

In the present disclosure MDBK cells were maintained at 37ºC, 5% CO2 in Dulbecco's modified Eagle's medium (D-MEM) (preferably InvitrogenTM, Paisley, UK) supplemented with 10% heat-inactivated horse serum (preferably InvitrogenTM, Paisley, UK), penicillin (100 U/ml) – streptomycin (100 µg/ml) (preferably Sigma-Aldrich, Steinheim, Germany) and sodium pyruvate (1 mM) (preferably InvitrogenTM, Paisley, UK). BT cells were maintained in the same conditions but supplemented with 10% FBS (preferably InvitrogenTM, Paisley, UK) and penicillin (100 U/ml) – streptomycin (100 µg/ml) (preferably Sigma-Aldrich, Steinheim, Germany). Suspensions of liver homogenate samples (1 ml) filtered at 0.2 µm (preferably Sarstedt, Nümbrecht, Germany) were inoculated on both cell cultures and these were subcultured once a week up to 7 and 2 passages of MDBK and BT cultures respectively. Uninoculated MDBK and BT cell cultures were also maintained in all passages as negative controls for all procedures.

Then, immunofluorescence evaluation of HCV NS3, NS4A and NS5 specific proteins in MDBK and BT cells was performed as previously described by Zhang *et al*. Cells inoculated with liver homogenate samples and the negative controls (uninoculated MDBK and BT cells) were subcultured 14 days post inoculation (second passage) and a drop of cell suspensions was placed on glass slides dots (6 mm) (preferably Poly Labo, Strasbourg, France), pretreated with poly-L-lysine solution (preferably Sigma-Aldrich, Steinheim, Germany). After incubation during 4 hours at 37ºC, 5% CO2, the slides were fixed with ice-cold acetone (preferably Merck, Darmstadt, Germany) and then treated with a blocking solution for 30 min. The slides were then incubated with mouse MAbs anti-HCV NS3, anti-HCV NS4A and anti-HCV NS5 (preferably all from Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:100) in blocking solution at room temperature for 60 min, and subsequently incubated with goat anti-mouse IgG-FITC (preferably Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:200) for 30 min. The slides were then counterstained with Evans blue (preferably Sigma-Aldrich, Steinheim, Germany) for 5 min, washed 3 times with 1x phosphate-buffered saline, mounted with florescence mounting solution and visualized in a fluorescence microscope (preferably BX51, equipped with a DP72 camera and software Cell-B, Olympus, Tokyo, Japan).

Moreover, for IEM, 20 µl of cell suspensions at 14 days post inoculation from each MDBK cell flasks with the liver samples and the negative controls (uninoculated cells) were processed according to procedures previously described by Falcon *et al*.. Cells were fixed in 1.25% glutaraldehyde (preferably Electron Microscopy sciences, Hatfield, USA) and 2% paraformaldehyde (preferably Merck, Darmstadt, Germany) in Tris Buffered Saline (preferably TBS), dehydrated and embedded in Epon resin (preferably TAAB, Berks, England). Ultrathin sections cut and placed on nickel grids (preferably TAAB, Berks, England) and were floated for 10 min on a drop of TBS in a moist chamber. The grids were then allowed to float for 30 min on a drop of 14.4 % sodium metaperiodate (preferably Sigma-Aldrich, Steinheim, Germany) and then washed with TBS. After blocking with 2% bovine serum albumin (BSA) (preferably Sigma-Aldrich, Steinheim, Germany) in TBS the grids were incubated for 60 min on a drop of the same primary MAbs used in IFA procedure diluted 1:50 in TBS-BSA overnight at 4ºC and then washed for several times. Then grids were incubated for 60 min in a drop of secondary anti-mouse IgG gold antibody (preferably Sigma-Aldrich, Steinheim, Germany) diluted 1:20 in TBS-BSA and then washed with TBS. Grids were counterstained with uranyl acetate and lead citrate as Reynolds methods and visualized in a transmission electron microscope operated at 60 kV by Jeol 1400 (preferably Tokyo, Japan) preferably with a CCD digital camera Orious 1100W Tokyo, Japan.

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In this embodiment MDBK cell line and primary BT cell cultures were inoculated with suspensions of liver homogenate samples from the wild, domestic rabbits and the hare as described above and tested by IFA as demonstrated in Figure 1. As MDBK cell line showed a better signal for European rabbit and *Lepus europaeus* endogenous HCV homolog fragments replication than BT primary cell cultures by IFA, they were selected for treatment by IEM (Figure 2). Negative controls (uninoculated MDBK and BT cells) were included and treated with the selected MAbs and no reaction was detected (Figure 1, 2).

In this disclosure, liver homogenates (1 ml, 10 % (w/v)) and DNA extracted from liver homogenates (300 µl) of domestic rabbit and hare were also inoculated on MDBK cells for several subcultures. MDBK cells were maintained as described above. DNA extraction with RNase A treatment for 90 min at 70ºC was performed from samples as described above. DNA, RNA and protein concentration was measured before and after treatment in a Qubit Fluorometer (preferably Invitrogen, Carlsbad, USA) according to the manufacturer instructions.

MDBK cells were inoculated with DNA samples and at the seventh passage (49 days post inoculation) were subjected to tree freeze/thaw cycles at -80ºC / room temperature. RNA from cell lysates (100 µl) with 5 µl of internal control (provided preferably with HCV Real-TM Quant kit) were extracted using the QIAamp Viral RNA Kit (preferably Qiagen, Hilden, Germany) following the manufacturer instructions. RNAs were eluted with 50 µl of buffer AVE, provided in the RNA extraction kit, and HCV RNA titers were measured by a HCV Real-TM Quant kit (preferably Sacace Biotechnologies Srl, Como, Italy) according to the manufacturer instructions in a StepOne™ Real-Time PCR System (preferably Applied Biosystems, Foster, California).

In an embodiment 20 ml of cell suspensions at 28 days (forth passage), 35 days (fifth passage) and 49 days (seventh passage) post inoculation from each MDBK cell flask with liver homogenates and DNA extracted from liver homogenates from domestic rabbit and hare, and the negative control (uninoculated MDBK cells) were processed for IEM as described above. Although herein HCV E2 specific protein was evaluated with mouse MAbs anti-HCV E2 (preferably Santa Cruz Biotechnology, Inc, Heidelberg, Germany).

In another embodiment DNA, RNA and proteins concentrations obtained for domestic rabbit and hare samples, before and after RNAse A treatment, using Qubit Fluorometer were measured (Table 3). Therefore, HCV RNA titers were determined using a Real time-PCR as described above and concentrations of 2.00E+04 and 5.00E+04 IU/ml for domestic rabbit and hare, respectively, were carried out. Uninoculated MDBK cells (negative control) were also tested and no amplification was detected.

Table 3.

| Animal | Before RNAse A treatment* | | | After RNAse A treatment | | |
|---|---|---|---|---|---|---|
| | DNA (ng / ml) | RNA (ng / ml) | Protein (µg / ml) | DNA (ng / ml) | RNA (ng / ml) | Protein (µg / ml) |
| DR | 92.800 | 82.400 | 3.000 | 2.500 | < 20 | > 26 |
| Hare | 90.600 | 82.000 | 2.860 | 640 | < 20 | < 1 |

DR – Domestic rabbit *(Oryctolagus cuniculus)*, WR – Wild rabbit *(Oryctolagus cuniculus.)*, Hare *(Lepus europaeu)*, * 1ml of liver homogenate 10 % (w/v).

Inoculated MDBK cells and negative control (uninoculated MDBK cells) were harvested at the fourth, fifth and seventh subcultures and tested by IEM. HCV E2 specific protein was detected in all tested samples, HCV-like particles were also detected and no reaction was detected in the negative control (Figure 4).

The disclosure also includes the use of HCV homolog synthetic nucleotides or peptides that infect and autonomously replicate in permissive bovine cell cultures. Synthetic nucleotides e.g., NS5B Pr3 (SEQ ID NO: 63): 5`-TATGAYACCCGCTGYTTTGACTC-3`, NS5B Pr4 (SEQ ID NO: 64): 5`-CNGARTAYCTVGTCATAGCCTC-3`, were inoculated on MDBK cells for 168 hours. MDBK cells were maintained as described above. MDBK cells harvested at the 168 were subjected to tree freeze/thaw cycles at -80ºC / room temperature. RNA from cell lysates (100 µl) with 5 µl of internal control (provided preferably with HCV Real-TM Quant kit) were extracted using the QIAamp Viral RNA Kit (preferably Qiagen, Hilden, Germany) following the manufacturer instructions. RNAs were eluted with 50 µl of buffer AVE, provided in the RNA extraction kit, and HCV RNA titers were measured by a HCV Real-TM Quant kit (preferably Sacace Biotechnologies Srl, Como, Italy) according to the manufacturer instructions in a StepOne™ Real-Time PCR System (preferably Applied Biosystems, Foster, California). HCV RNA titer of 1.50E+04 IU/ml was determined using a Real Time-PCR as described above.

The disclosure also includes Human HCV isolates that infect and replicate in permissive bovine cell cultures. Serum sample from a patient with HCV-1a infection, gently provided by the Hospital de Santo António, Porto, Portugal, was inoculated on MDBK cells maintained as described above. A T25 cell culture flask with 80% confluence of MDBK cells was inoculated with 1 ml of the sample (3.76E+05 IU/ml) for 16 hours at 37ºC and 5%. After 16 hours, the T25 flask was subcultured and 1 ml of the cell suspension was placed per well in a 24-well plate (two 24-well plates for the virus and one plate for negative control). Duplicates wells of HCV-1a and the negative control (MDBK cells) were maintained at 37ºC and 5% $CO_2$ for 336 hours. Duplicate wells with HCV-1a treated with antivirals interferon-α2b (500 IU/mL), Ribavirin (20 µM) and Danoprevir (100 nM) or combinations (interferon-α2b (500 IU/mL) + Ribavirin (20 µM), interferon-

α2b (500 IU/mL) + Danoprevir (100nM), Ribavirin (20 μM) + Danoprevir (100 nM) and interferon-α2b (500 IU/mL) + Ribavirin (20 μM) + Danoprevir (100nM)) were also included and maintained at 37ºC and 5% $CO_2$ for 96 hours. Duplicate wells, supernatants and cells, were harvested at 6, 48, 72, 96, 144 and 336 hours and 6, 48, 72, 96 hours from HCV-1a and treatments, respectively, for HCV RNA quantification by Real Time-PCR and immunofluorescence analysis were also performed at some of the defined time points.

Then, duplicates wells from MDBK cells and supernatants from negative controls, HCV-1a and with treatments harvested at the defined time points were subjected to tree freeze/thaw cycles at -80ºC / room temperature and RNA extraction and HCV RNA titers were performed as described above.

In this disclosure, immunofluorescence evaluation of HCV NS3 and NS5 specific proteins from cells inoculated with HCV-1a genotype at 16 and 96 hours and 16, 48 and 96 hours for HCV NS3 and HCV NS5 specific proteins, respectively, for HCV-1a treated with different antivirals (interferon-α2b (500 IU/mL), Ribavirin (20 μM) and Danoprevir (100 nM) or combinations (interferon-α2b (500 IU/mL) + Ribavirin (20 μM), interferon-α2b (500 IU/mL) + Danoprevir (100nM), Ribavirin (20 μM) + Danoprevir (100 nM) and interferon-α2b (500 IU/mL) + Ribavirin (20 μM) + Danoprevir (100nM)) at 96h for HCV NS3 and at 48 and 96h for HCV NS5 and the negative controls (uninoculated MDBK cells) was performed. From the harvested duplicate wells with inoculated HCV-1a or from duplicate wells with treatment a drop of cell suspensions was placed on glass slides dots (6 mm) (preferably Poly Labo, Strasbourg, France), pretreated with poly-L-lysine solution (preferably Sigma-Aldrich, Steinheim, Germany). After incubation during 4 hours at 37ºC, 5% $CO_2$, the slides were fixed with ice-cold acetone (preferably Merck, Darmstadt, Germany). The slides were then incubated with mouse MAbs anti-HCV NS3 and anti-HCV NS5 (preferably all from Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:10) in PBS at 37ºC for 60 min, and subsequently incubated with goat anti-mouse IgG-FITC (preferably Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:40) for 30 min at 37ºC. The slides were then counterstained with Evans blue (preferably Sigma-Aldrich, Steinheim, Germany) for 3 min, washed with $ddH_2O$, mounted with florescence mounting solution and visualized in a fluorescence microscope (preferably Axio Imager A1, equipped with a Axiocam MRC camera and software AxioVision 4.8, Zeiss, Germany).

In this embodiment inoculated MDBK cells with HCV genotype -1a, harvested at equal or at different time points and treated or not treated with individual or combined antivirals as described above were tested by IFA using mouse MAbs anti-HCV NS3 and anti-HCV NS5, specific immunostaining for the two selected antibodies could be demonstrated (Figure 5). Negative

controls (uninoculated MDBK) were included and treated with the selected MAbs and no reaction was detected (Figure 5).

In another embodiment, the HCV RNA titer from the -1a genotype and the -1a genotype with antiviral treatments as described above on MDBK cell cultures was studied and kinetic experiments were carried out (Figure 6).

EXAMPLES

Below are examples and preparations given to enable those skilled in the art to more clearly understand and to practice the present subject matter. They are offered for illustrative purposes only, and should not be considered as limiting the scope of disclosure in any way.

EXAMPLE 1

Materials and Methods

Samples: Samples (1 ml) from domestic rabbits, wild rabbits and hare, e.g. liver homogenates, serum, white blood cells, body fluids were inoculated on MDBK and BT cells.

Cells: Madin-Darby bovine kidney (MDBK) epithelial cell line were maintained at 37ºC, 5% CO2 in Dulbecco`s modified Eagle medium (D-MEM) (preferably InvitrogenTM, Paisley, UK) supplemented with 10% heat-inactivated horse serum (preferably InvitrogenTM, Paisley, UK), penicillin (100 U/ml) – streptomycin (100 mg/ml) (preferably Sigma-Aldrich, Steinheim, Germany) and sodium pyruvate (1 mM) (preferably InvitrogenTM, Paisley, UK) in tissue culture flasks, e.g. T25, T150. Uninoculated MDBK cell cultures were also maintained in all passages as negative controls for all procedures.

Cells: Primary BT cells were obtained from bovine testis collected in a local cattle slaughterhouse (preferably Famalicão, Portugal). Testicles from bovine fetuses recovered at an abattoir from pregnant cows, were excised and the capsule was removed. The tissue was chopped with sterile scissors into pieces approximately 1 $mm^3$ and washed with phosphate buffered saline (PBS) until rinse water is clear. The chopped tissue was added to a flask with solution of 0.25% trypsin (preferably Sigma-Aldrich, Steinheim, Germany) at 37ºC, a magnetic stirring bar and mixed at low speed for 10 min. New 0.25% trypsin solutions were added until large digested tissue fragments

settle in the flask. Final digest was filtered through three layers sterile gauze to an iced flask for 3 times and centrifuged for 10 min at 6000xg. Supernatant was discarded and cells were resuspended in tissue culture flasks, e.g. T25, T150, with D-MEM (preferably InvitrogenTM, Paisley, UK) supplemented with 10% FBS (preferably InvitrogenTM, Paisley, UK), penicillin (100 U/ml) – streptomycin (100 mg/ml) (preferably Sigma-Aldrich, Steinheim, Germany) at 37ºC, 5% CO2. BT cells were maintained in the same conditions or stored in liquid nitrogen. Uninoculated BT cell cultures were also maintained in all passages as negative controls for all procedures.

RNA Extraction: Total RNA was extracted from samples using TRI Reagent LS (preferably Sigma-Aldrich, Steinheim, Germany) according to the manufacturer instructions.

DNA Extractions: Genomic DNA was extracted from samples using the QIAamp DNA blood kit (preferably Qiagen, Hilden, Germany) according to the manufacturer instructions.

cDNA Synthesis: From the RNA extracts cDNA was synthesized using random hexamers (preferably Amersham, New Jersey, USA). RNA (5 ml) of RNA was mixed with 2 ml of diethyl pyrocarbonate (preferably Sigma-Aldrich, Steinheim, Germany) water and 1 ml (0.02 U) of random hexamer, denaturated at 65ºC for 10 min and then cooled on ice for 10 min. Thereafter, the hexamers were extended for 90 min at 37ºC by 1 ml Moloney murine leukaemiavirus reverse transcriptase (200 U) (preferably InvitrogenTM, Paisley, UK) in the presence of 2.0 ml dNTPs (10 mM), 5 ml 1st standard buffer and 1 ml RNAse inhibitor (preferably Roche, Mannheim, Germany). The enzyme was then inactivated for 5 min at 95ºC and the cDNA was immediately used or kept at -20ºC until further processed.

RT-PCR: Using cDNA, RT-PCR were performed targeting the specific HCV structural and NS proteins, core, envelope glycoprotein's E1/E2, NS5A/B and NS5B in a thermocycler C 1,000 (Bio-Rad, California, USA). The PCR mix (25 ml) contained 2.5 ml of 106 PCR buffer, 0.6 ml dNTPs (10 mM), 10 pmol of each primer, 2.5 ml MgCl2 (25 mM) and 1 U Taq DNA polymerase (Fermentas, St. Leon-Rot, Germany). PCR cycling conditions were performed as described above. The amplified products were analyzed on a 1.2% (w/v) agarose gel stained with gel red™ (Biotium, California, USA) and visualized under UV light (Bio-Rad, California, USA).

PCR: Using DNA, PCR were performed as RT-PCR and the same primers were used.

Sequencing of the Amplified Fragments: RT-PCR and PCR amplicons were directly sequenced after being purified using a Qiagen kit (preferably Qiagen, QIAquickH Gel Extraction Kit, Crawley, UK) using the same primers.

Proteins Extraction: Proteins were extracted from samples following preferably the Alliance for Cellular Signaling (AFCS) protocol.

Proteins Quantification: Protein concentration was determined in a Qubit Fluorometer (preferably Invitrogen, Carlsbad, USA) according to the manufacturer instructions.

Proteomic Analysis: After extraction, samples proteins were separated by 12% SDS-PAGE, performed by Laemmli method, using 30 mg of total protein in a Mini-Protean 3 Cell (preferably Bio-Rad, California, USA) system. After SDS-PAGE separation, proteins were Coomassie blue stained (preferably Imperial Protein stain, Thermo Scientific, Rockford, USA).The protein bands of interest were reduced, alkylated and in gel digested with trypsin accordingly to trypsin manufacturer's suggested protocol (preferably Promega, USA). The resulting peptides were concentrated with ZipTips (preferably Millipore, USA) in agreement with manufacturer's instructions and eluted into the MALDI plate using alpha-cyano 4-hydroxycinnamic acid as a matrix for MALDITOF/TOF-MS/MS analysis. MS and MS/MS peptide mass spectra were acquired with a MALDI-TOF/TOF 4700 Proteomics Analyzer (preferably ABSCIEX, USA). Peptide mass spectra were obtained in reflector positive mode for a mass window of 700–4000 Da. Some peptides were selected for MS/MS fragmentation by collision induced dissociation (CID) in MS/ MS positive mode. Proteins were identified using the combined information of MS and MS/MS spectra by the GPS (preferably Global Proteome Server) Explorer Software v3.6 (preferably ABSCIEX, USA) which integrates the Mascot protein search engine v2.1.04 (preferably Matrix Science, UK) configured to perform searches at the Swiss-Prot/ UnitProt database.

Immunofluorescence Assay (IFA): Immunofluorescence evaluation of HCV NS3, NS4A and NS5 specific proteins in MDBK and BT cells was performed. Cells inoculated with samples and the negative controls (uninoculated MDBK and BT cells) were subcultured 14 days post inoculation (second passage) and a drop of cell suspensions was placed on glass slides dots (6 mm) (preferably Poly Labo, Strasbourg, France), pretreated with poly-L-lysine solution (preferably Sigma-Aldrich, Steinheim, Germany). After incubation during 4 hours at 37ºC, 5% $CO_2$, the slides were fixed with ice-cold acetone (preferably Merck, Darmstadt, Germany) and then treated with a

blocking solution for 30 min. The slides were then incubated with mouse MAbs anti-HCV NS3, anti-HCV NS4A and anti-HCV NS5 (preferably all from Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:100) in blocking solution at room temperature for 60 min, and subsequently incubated with goat anti-mouse IgG-FITC (preferably Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:200) for 30 min. The slides were then counterstained with Evans blue (preferably Sigma-Aldrich, Steinheim, Germany) for 5 min, washed 3 times with 1x PBS mounted with florescence mounting solution and visualized in a fluorescence microscope (preferably BX51, equipped with a DP72 camera and software Cell-B, Olympus, Tokyo, Japan).

Immunogold Electron Microscopy (IEM): For IEM, 20 ml of cell suspensions at 14 days post inoculation from each MDBK cell flask with the samples and the negative control (uninoculated MDBK cells) were processed. Cells were fixed in 1.25% glutaraldehyde (preferably Electron Microscopy sciences, Hatfield, USA) and 2% paraformaldehyde (preferably Merck, Darmstadt, Germany) in Tris Buffered Saline (TBS), dehydrated and embedded in Epon resin (preferably TAAB, Berks, England). Ultrathin sections cut and placed on nickel grids (preferably TAAB, Berks, England) and were floated for 10 min on a drop of TBS in a moist chamber. The grids were then allowed to float for 30 min on a drop of 14.4% sodium metaperiodate (preferably Sigma-Aldrich, Steinheim, Germany) and then washed with TBS. After blocking with 2% BSA (preferably Sigma-Aldrich, Steinheim, Germany) in TBS the grids were incubated for 60 min on a drop of the same primary MAbs used in IFA procedure diluted 1:50 in TBS-BSA overnight at 4ºC and then washed for several times. Then grids were incubated for 60 min in a drop of secondary anti-mouse IgG gold antibody (preferably Sigma-Aldrich, Steinheim, Germany) diluted 1:20 in TBS-BSA and then washed with TBS. Grids were counterstained with uranyl acetate and lead citrate as Reynolds methods and visualized in a transmission electron microscope operated at 60 kV by Jeol 1400 (preferably Tokyo, Japan) with a CCD digital camera Orious 1100W Tokyo, Japan.

Results

RT-PCR: Core, E1/E2, NS5A/B and NS5B HCV genomic proteins were successfully amplified from samples, e.g. liver homogenates from domestic rabbits, wild rabbits and hare, using above described methods for RT-PCR. NS5A/B HCV proteins were also amplified by RT-PCR from samples, e.g. white blood cells and serum from domestic rabbits and body fluids from wild rabbits. Amplified fragments were detected only from white blood cells.

PCR: Core, E1/E2, NS5A/B and NS5B HCV genomic proteins were successfully amplified from samples, e.g. liver homogenates from domestic rabbits, wild rabbits and hare, using above described methods for PCR.

Sequencing: Sequencing of RT-PCR and PCR amplified regions was performed. Alignments and tblastn searches were conducted as a query in the HCV database and whole-genome shotgun in rabbit genome resources at GenBank, NCBI. Retrieved fragment sequences from core, E1/E2, NS5A/B and NS5B HCV specific proteins were identified (Table 1).

Analysis of Rabbit and Hare by MALDI-TOF/TOF: The visible proteins, e.g. from liver homogenates, bands separated by SDS-PAGE and stained with Coomassie Blue selected on the basis of their molecular weight and that could harbor proteins matching the core, envelope glycoprotein's E1 and E2, serine protease NS3, NS4B, NS5A and NS5B HCV genomic proteins, were excised from the gel, and analyzed by MALDITOF/TOF-MS/MS. For each digested sample, the MS and MS/MS spectra were combined for the MASCOT database search. With the matched MS/MS spectra of domestic, wild rabbits and hare to HCV sequences deposited at Swiss-Prot/UniProt protein database a total of 34 peptide sequences were identified, such as core, envelope glycoprotein's E1 and E2, protease NS2–3, serine protease NS3, NS5A and RdRp-NS5B HCV specific proteins (Table 2). For domestic rabbit we were able to identify 16 peptide sequences that match core, envelope glycoprotein's E1 and E2, protease NS2–3, serine protease NS3, NS5A and RdRp-NS5B HCV specific proteins (Table 2). In wild rabbits, 12 peptide sequences that match to the core, serine protease NS3, NS5A and RdRp-NS5B HCV specific proteins were identified (Table 2). From hare we were able to identify 6 peptide sequences that match core, envelope glycoprotein E2, protease NS2–3, NS5A and RdRp-NS5B HCV specific proteins (Table 2). Spectra generated by MS/MS analysis from all identified peptide sequences can be observed in Figure 3.

IFA of Rabbit and Hare in Bovine Cell Cultures: Inoculated cells, MDBK and BT, were harvested at the second subculture (14 days post infection) and tested by IFA using mouse MAbs anti-HCV NS3, anti-HCV NS4A and anti-HCV NS5, specific immunostaining for the three selected antibodies could be demonstrated (Figure 1). Negative controls (uninoculated MDBK and BT cells) were included and treated with the selected MAbs and no reaction was detected (Figure 1).

IEM of Rabbit and Hare in Bovine Cell Cultures: As inoculated MDBK cells showed a better signal in IFA procedure, they were selected for treatment by IEM, immunofluorescence was obtained for tested samples (Figure 2). Negative controls (uninoculated MDBK cells) were included and treated with the selected MAbs and no reaction was detected (Figure 2).

EXAMPLE 2

Materials and Methods

Samples: Samples, e.g. liver homogenates (1 ml, 10 % (w/v)), DNA extracted from liver homogenates (300 µl) of domestic rabbit and hare were inoculated on MDBK cells for several subcultures.

Cells: MDBK were maintained as described in example 1.

DNA Extractions: DNA was extracted from samples, e.g. liver homogenates, using the same kit as described in example 1. The samples were treated with RNase A for 90 min at 70ºC. DNA, RNA and protein concentration was measured before and after treatment preferably in a Qubit Fluorometer (preferably Invitrogen, Carlsbad, USA) according to the manufacturer instructions.

RNA Extraction: MDBK cells inoculated with DNA samples, at seventh passage (49 days post inoculation) were subjected to tree freeze/thaw cycles at -80ºC / room temperature. RNA from cell lysates (100 µl) with 5 µl of internal control (provided preferably with HCV Real-TM Quant kit) were extracted using the QIAamp Viral RNA Kit (preferably Qiagen, Hilden, Germany) following the manufacturer instructions. RNAs were eluted with 50 µl of buffer AVE, provided in the RNA extraction kit.

HCV RNA Titers: HCV RNA titers from RNA extracted samples were measured by a HCV Real-TM Quant kit (preferably Sacace Biotechnologies Srl, Como, Italy) according to the manufacturer instructions in a StepOne™ Real-Time PCR System (preferably Applied Biosystems, Foster, California).

Immunogold Electron Microscopy (IEM): For IEM, 20 ml of cell suspensions at 28 days (forth passage), 35 days (fifth passage) and 49 days (seventh passage) post inoculation from each MDBK cell flask with samples, e.g. liver homogenates, DNA extracted from liver homogenates from domestic rabbit and hare, and the negative control (uninoculated MDBK cells) were processed as described in example 1.HCV E2 specific protein was evaluated with mouse MAbs anti-HCV E2 (preferably Santa Cruz Biotechnology, Inc, Heidelberg, Germany) and anti-mouse IgG gold antibody (preferably Sigma-Aldrich, Steinheim, Germany) as described in example 1 (Figure 4).

Results

DNA, RNA and Proteins present in samples: DNA, RNA and proteins concentrations obtained for tested samples could be seen in Table 3.

HCV RNA titers: MDBK cell cultures inoculated with samples, e.g. DNA extracted from liver homogenates, were harvested 49 days post infection and tested for HCV RNA titers using a Real time-PCR as described above and concentrations of 2.00E+04 and 5.00E+04 IU / ml for domestic rabbit and hare, respectively, were carried out (data not shown). Uninoculated MDBK cells (negative control) were also tested and no amplification was detected. These results suggest MDBK cells as a new efficient model for HCV replication.

IEM of Rabbit and Hare samples in MDBK Cell Cultures: Inoculated MDBK cells and negative control (uninoculated MDBK cells) were harvested at the fourth, fifth and seventh subcultures and tested by IEM. HCV E2 specific protein was detected in all tested samples, HCV-like particles were also detected and no reaction was detected in the negative control (Figure 4).These results demonstrate the capacity of the tested samples to infect, replicate and generate entire HCV-like particles in MDBK cell cultures.

EXAMPLE 3

Materials and Methods

Samples: Samples, e.g. serum from a patient with HCV-1a infection, gently provided by the Hospital de Santo António, Porto, Portugal, were inoculated on MDBK cells.

Cells: MDBK were maintained as described in example 1. A T25 cell culture flask with 80% confluence of MDBK cells was inoculated with 1 ml of the sample (3.76E+05 IU/ml) for 16 hours at 37ºC and 5%. After 16 hours, the T25 flask was subcultured and 1 ml of the cell suspension was placed per well in a 24-well plate (two 24-well plates for the virus and one plate for negative control). Duplicates wells of HCV-1a and the negative control (MDBK cells) were maintained at 37ºC and 5% $CO_2$ for 336 hours. Duplicate wells with HCV-1a treated with antivirals interferon-α2b (500 IU/mL), Ribavirin (20 µM) and Danoprevir (100 nM) or combinations (interferon-α2b (500 IU/mL) + Ribavirin (20 µM), interferon-α2b (500 IU/mL) + Danoprevir (100nM), Ribavirin (20 µM) + Danoprevir (100 nM) and interferon-α2b (500 IU/mL) + Ribavirin (20 µM) + Danoprevir (100nM)) were also included and maintained at 37ºC and 5% $CO_2$ for 96 hours. Duplicate wells, supernatants and cells, were harvested at 6, 48, 72, 96, 144 and 336 hours and 6, 48, 72, 96 hours from HCV-1a and treatments, respectively, for HCV RNA quantification by Real time-PCR and immunofluorescence analysis were also performed at some of the defined time points.

Then, duplicates wells from MDBK cells and supernatants, negative controls, HCV-1a and with treatments harvested at the defined time points were subjected to RNA extraction and HCV RNA titers as described in above.

In this disclosure, immunofluorescence evaluation of HCV NS3 and NS5 specific proteins from cells inoculated with HCV-1a genotype at 16 and 96 hours and 16, 48 and 96 hours for HCV NS3 and HCV NS5 specific proteins, respectively, for HCV-1a treated with different antivirals (interferon-α2b (500 IU/mL), Ribavirin (20 µM) and Danoprevir (100 nM) or combinations (interferon-α2b (500 IU/mL) + Ribavirin (20 µM), interferon-α2b (500 IU/mL) + Danoprevir (100nM), Ribavirin (20 µM) + Danoprevir (100 nM) and interferon-α2b (500 IU/mL) + Ribavirin (20 µM) + Danoprevir (100nM)) at 96h for HCV NS3 and at 48 and 96h for HCV NS5 and the negative controls (uninoculated MDBK cells) was performed. From the harvested duplicate wells with inoculated HCV-1a or from duplicate wells with treatment a drop of cell suspensions was placed on glass slides dots (6 mm) (preferably Poly Labo, Strasbourg, France), pretreated with poly-L-lysine solution (preferably Sigma-Aldrich, Steinheim, Germany). After incubation during 4 hours at 37ºC, 5% $CO_2$, the slides were fixed with ice-cold acetone (preferably Merck, Darmstadt, Germany). The slides were then incubated with mouse MAbs anti-HCV NS3 and anti-HCV NS5 (preferably all from Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:10) in PBS at 37ºC for 60 min, and subsequently incubated with goat anti-mouse IgG-FITC (preferably Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:40) for 30 min at 37ºC. The slides were then counterstained with Evans blue (preferably Sigma-Aldrich, Steinheim, Germany) for 3 min,

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washed with $ddH_2O$, mounted with florescence mounting solution and visualized in a fluorescence microscope (preferably Axio Imager A1, equipped with a Axiocam MRC camera and software AxioVision 4.8, Zeiss, Germany).

In this embodiment inoculated MDBK cells with HCV genotype -1a, harvested at equal or at different time points and treated or not treated with individual or combined antivirals as described above were tested by IFA using mouse MAbs anti-HCV NS3 and anti-HCV NS5, specific immunostaining for the two selected antibodies could be demonstrated (Figure 5). Negative controls (uninoculated MDBK) were included and treated with the selected MAbs and no reaction was detected (Figure 5).

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The following claims set out particular embodiments of the present disclosure.

## C L A I M S

1. Isolated fragments for an in vitro model of HCV replication in cell lines, said fragments having the sequence with least 95% of homology of at least one of SEQ ID No. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 36, 37, 38, 39, 40, 41, 42 , 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 with the capability to replicate autonomously when inoculated on a cell line permissible to the said sequences.
2. Isolated fragments according to the previous claim wherein said fragments are from rabbit or hare.
3. Isolated fragments according to the previous claim wherein said cell lines permissible are bovine cell cultures, preferably MDBK or BT.
4. Cell cultures for an in vitro model of HCV replication, having at least one of the isolated fragment sequences having at least one of the isolated nucleotide or peptide sequences with at least 95% of homology of at least one of SEQ ID No. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42 , 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 with the capability to replicate autonomously when inoculated on the cells permissible to the said sequences.
5. Cell cultures according to the previous claim wherein said cell cultures permissible are bovine cell cultures, preferably MDBK or BT.
6. Cell cultures according to the previous claims for use in medicine.
7. Use of cell cultures described in any one of the previous claims as in vitro models for HCV replication.
8. A method for evaluating the viability or metabolism in cell cultures for develop diagnostic/therapeutic methods for HCV after contact with a test substance, cell or protein which comprises the following steps:
    - contacting a test substance, cell or protein with cell cultures for an in vitro model of HCV replication cells described in any claim 3-5;
    - analyzing the viability or metabolism of the cell culture.
9. The method according to the previous claim wherein the test substance is anti-nucleases, protease inhibitors, cell receptors.

10. A kit for evaluating the viability or metabolism in cell cultures for develop diagnostic/therapeutic methods for HCV after contact with a test substance, comprising cell cultures for an in vitro model of HCV replication cells described in any claim 1-5.

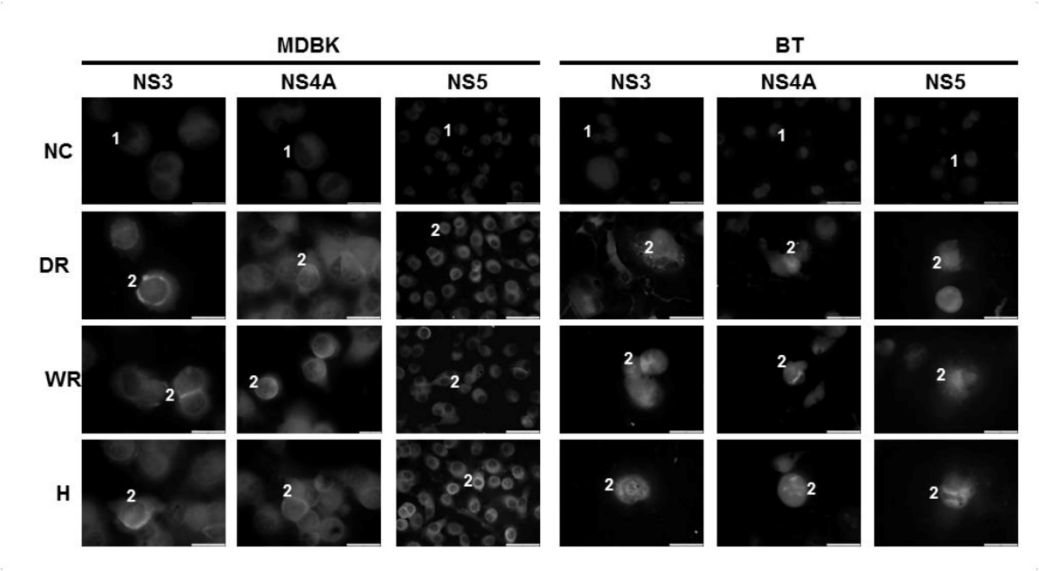



**Figure 1**

1

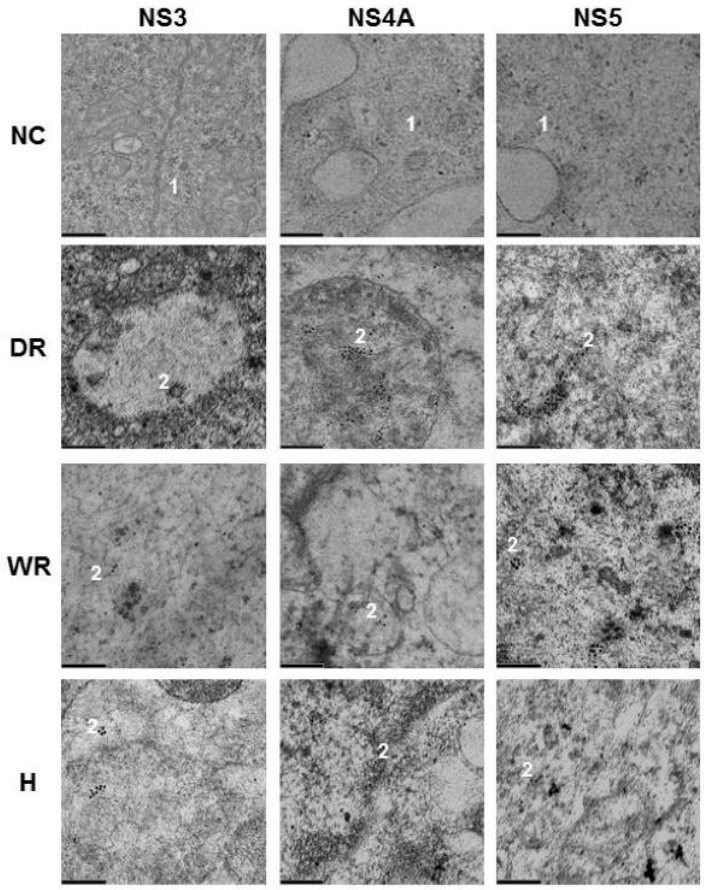
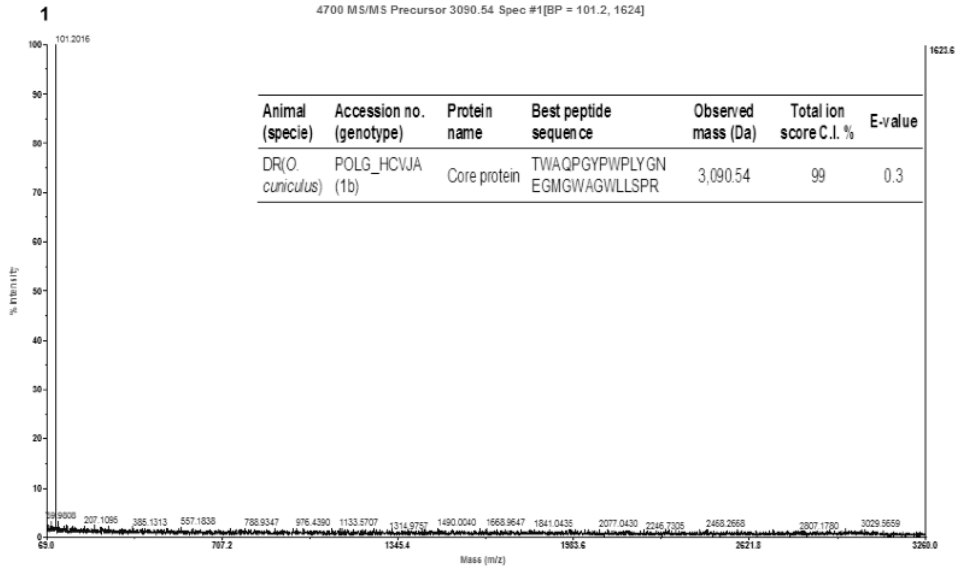



**Figure 2**

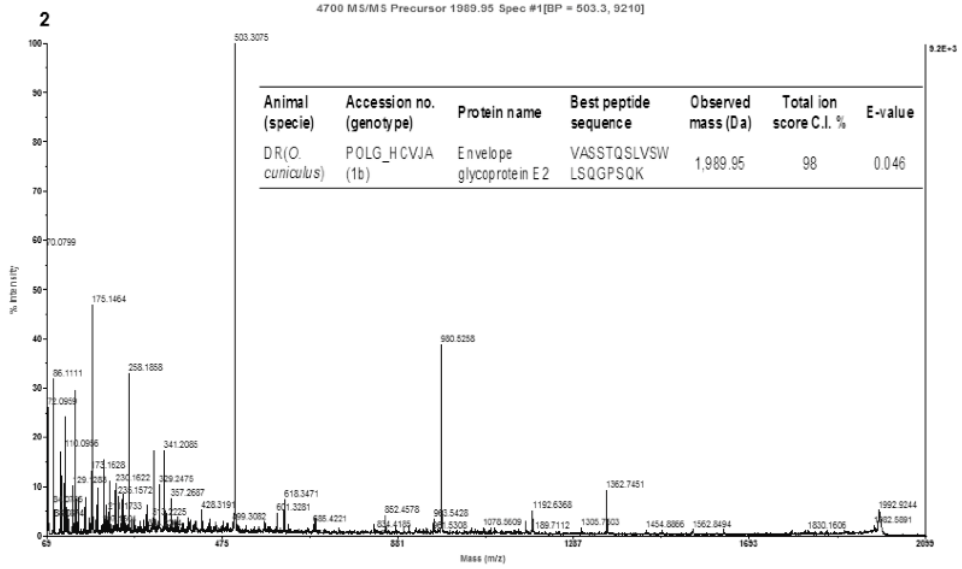
2

1



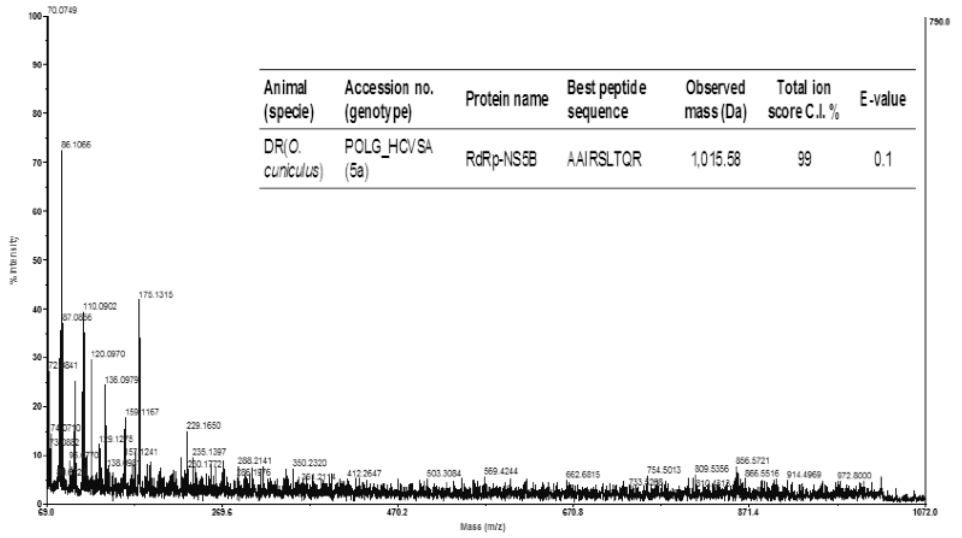

| Animal (specie) | Accession no. (genotype) | Protein name | Best peptide sequence | Observed mass (Da) | Total ion score C.I. % | E-value |
|---|---|---|---|---|---|---|
| DR(*O. cuniculus*) | POLG_HCVJA (1b) | Core protein | TWAQPGYPWPLYGN EGMGWAGWLLSPR | 3,090.54 | 99 | 0.3 |

2




| Animal (specie) | Accession no. (genotype) | Protein name | Best peptide sequence | Observed mass (Da) | Total ion score C.I. % | E-value |
|---|---|---|---|---|---|---|
| DR(*O. cuniculus*) | POLG_HCVJA (1b) | Envelope glycoprotein E2 | VASSTQSLVSW LSQGPSQK | 1,989.95 | 98 | 0.046 |

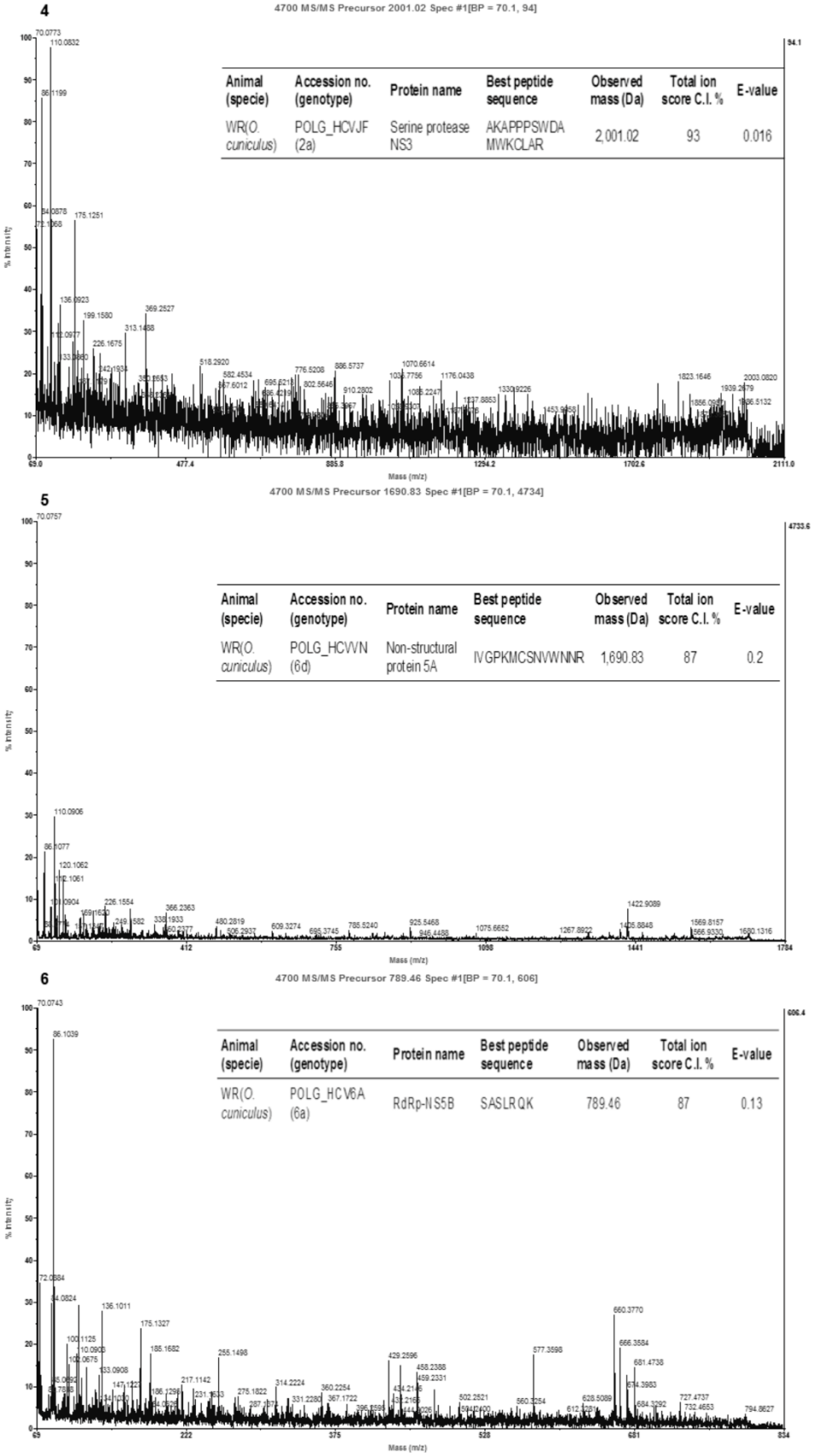
3




| Animal (specie) | Accession no. (genotype) | Protein name | Best peptide sequence | Observed mass (Da) | Total ion score C.I. % | E-value |
|---|---|---|---|---|---|---|
| DR(*O. cuniculus*) | POLG_HCVSA (5a) | RdRp-NS5B | AAIRSLTQR | 1,015.58 | 99 | 0.1 |

**Figure 3a**

3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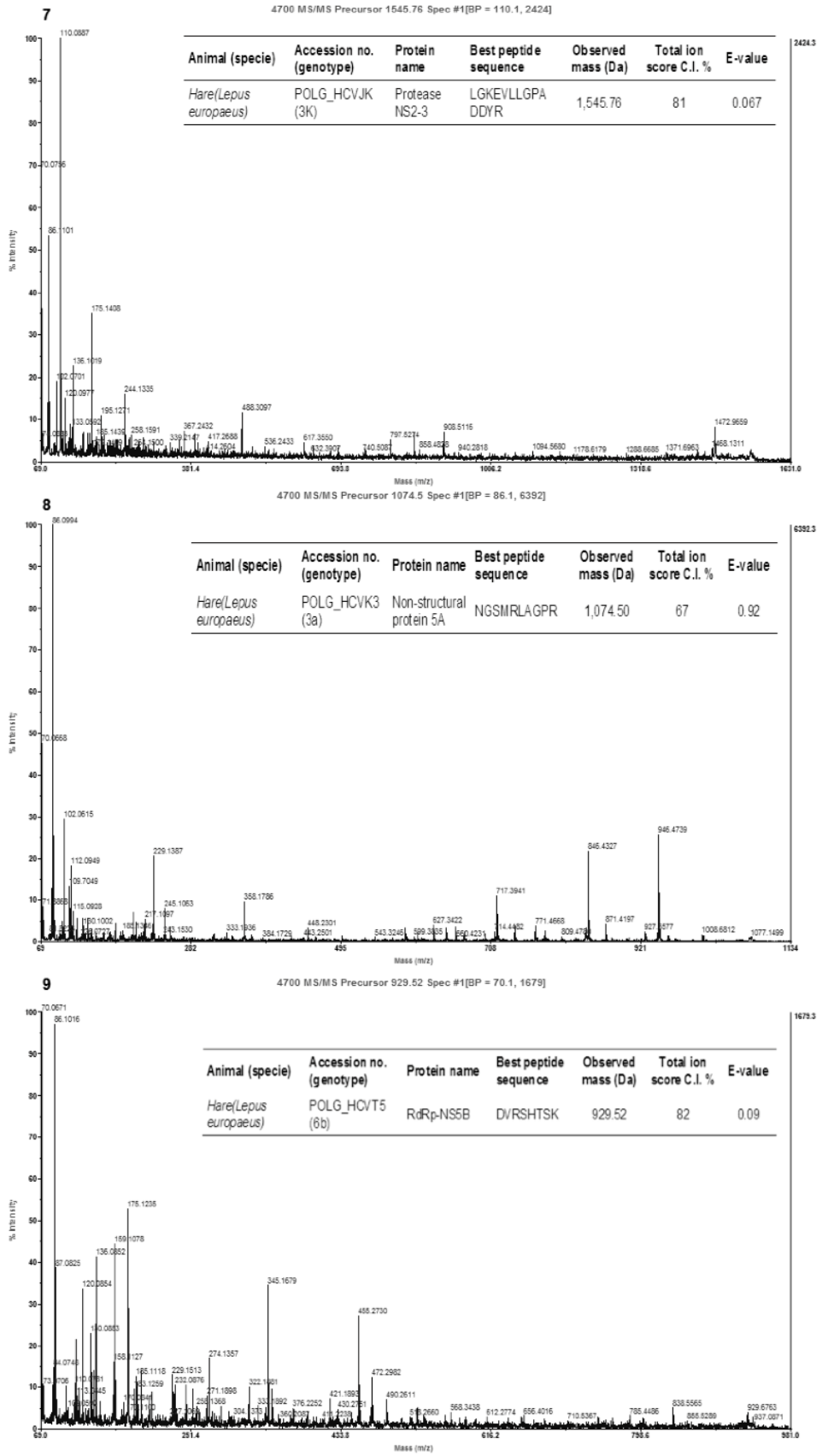
4700 MS/MS Precursor 2001.02 Spec #1[BP = 70.1, 94]
Animal (specie) | Accession no. (genotype) | Protein name | Best peptide sequence | Observed mass (Da) | Total ion score C.I. % | E-value
WR(O. cuniculus) | POLG_HCVJF (2a) | Serine protease NS3 | AKAPPPSWDA MWKCLAR | 2,001.02 | 93 | 0.016
% Intensity
Mass (m/z)
5
4700 MS/MS Precursor 1690.83 Spec #1[BP = 70.1, 4734]
Animal (specie) | Accession no. (genotype) | Protein name | Best peptide sequence | Observed mass (Da) | Total ion score C.I. % | E-value
WR(O. cuniculus) | POLG_HCVVN (6d) | Non-structural protein 5A | IVGPKMCSNVWNNR | 1,690.83 | 87 | 0.2
% Intensity
Mass (m/z)
6
4700 MS/MS Precursor 789.46 Spec #1[BP = 70.1, 606]
Animal (specie) | Accession no. (genotype) | Protein name | Best peptide sequence | Observed mass (Da) | Total ion score C.I. % | E-value
WR(O. cuniculus) | POLG_HCV6A (6a) | RdRp-NS5B | SASLRQK | 789.46 | 87 | 0.13
% Intensity
Mass (m/z)

**Figure 3b**

4




| Animal (specie) | Accession no. (genotype) | Protein name | Best peptide sequence | Observed mass (Da) | Total ion score C.I. % | E-value |
|---|---|---|---|---|---|---|
| *Hare(Lepus europaeus)* | POLG_HCVJK (3K) | Protease NS2-3 | LGKEVLLGPA DDYR | 1,545.76 | 81 | 0.067 |

| Animal (specie) | Accession no. (genotype) | Protein name | Best peptide sequence | Observed mass (Da) | Total ion score C.I. % | E-value |
|---|---|---|---|---|---|---|
| *Hare(Lepus europaeus)* | POLG_HCVK3 (3a) | Non-structural protein 5A | NGSMRLAGPR | 1,074.50 | 67 | 0.92 |

| Animal (specie) | Accession no. (genotype) | Protein name | Best peptide sequence | Observed mass (Da) | Total ion score C.I. % | E-value |
|---|---|---|---|---|---|---|
| *Hare(Lepus europaeus)* | POLG_HCVT5 (6b) | RdRp-NS5B | DVRSHTSK | 929.52 | 82 | 0.09 |

**Figure 3c**

5

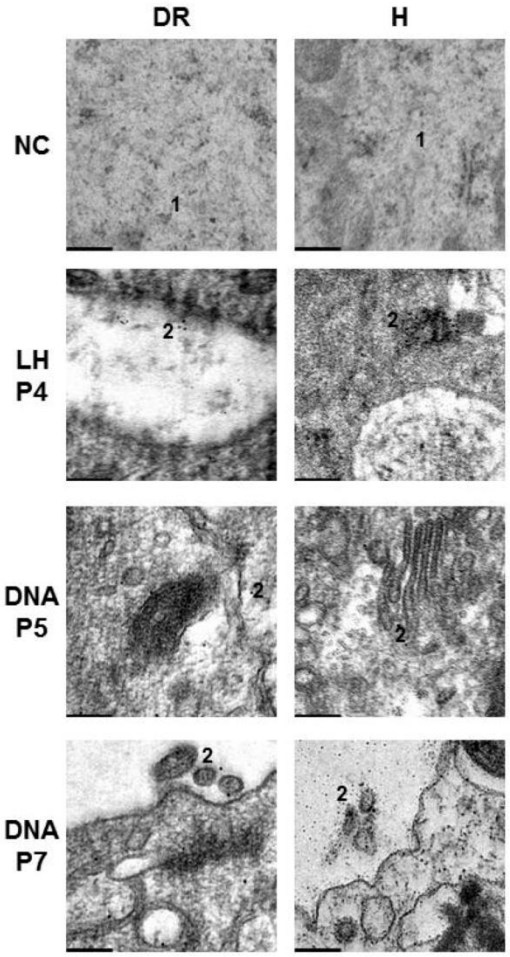



**Figure 4**

6

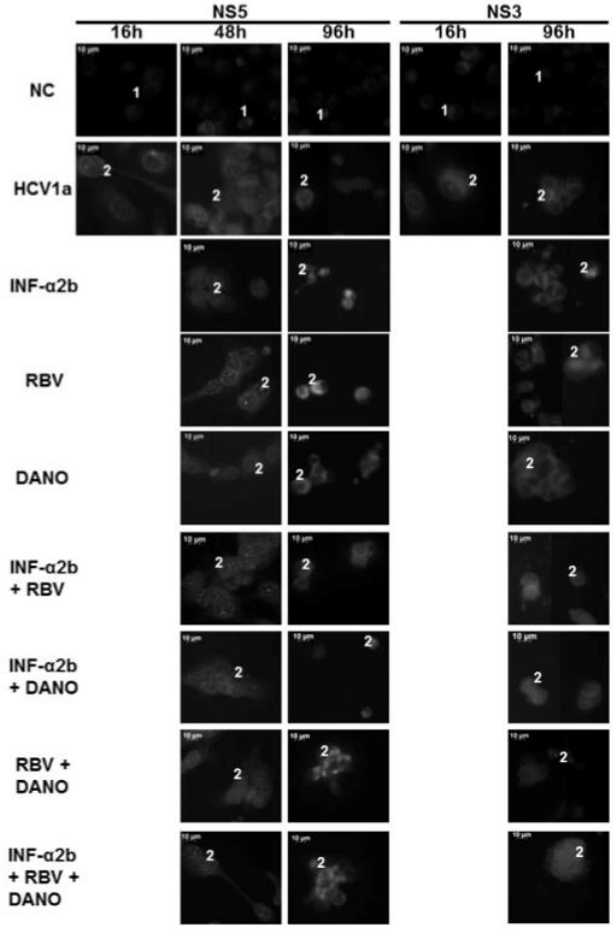



**Figure 5**

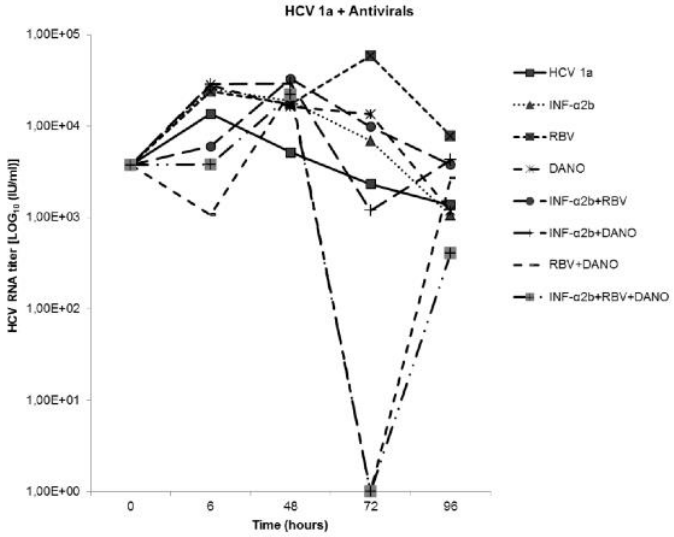



**Figure 6**

7

## 3.2 - Endogenous Hepatitis C Virus Homolog Fragments in European Rabbit and Hare Genomes Replicate in Cell Culture

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# Endogenous Hepatitis C Virus Homolog Fragments in European Rabbit and Hare Genomes Replicate in Cell Culture

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## Abstract

Endogenous retroviruses, non-retroviral RNA viruses and DNA viruses have been found in the mammalian genomes. The origin of Hepatitis C virus (HCV), the major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma in humans, remains unclear since its discovery. Here we show that fragments homologous to HCV structural and non-structural (NS) proteins present in the European rabbit (*Oryctolagus cuniculus*) and hare (*Lepus europaeus*) genomes replicate in bovine cell cultures. The HCV genomic homolog fragments were demonstrated by RT-PCR, PCR, mass spectrometry, and replication in bovine cell cultures by immunofluorescence assay (IFA) and immunogold electron microscopy (IEM) using specific MAbs for HCV NS3, NS4A, and NS5 proteins. These findings may lead to novel research approaches on the HCV origin, genesis, evolution and diversity.

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## Introduction

Viruses may represent genetic elements that gained the ability to move between cells, or represent previously free-living organisms that became parasites, or they may be the precursors of life [1]. The only group of viruses known to have left a fossil record in the form of endogenous provirus's are the retroviruses. There are studies describing that 8% of the human genome is made up of these elements [2,3]. Many other viruses, like non-retroviral RNA viruses, are known to generate DNA forms of their own genomes during replication [4]. Recently a handful of endogenous retroviruses, non-retroviral RNA viruses and DNA viruses have been found in mammalian genomes. Among these, Human Retrovirus 5 and endogenous lentivirus were found to be present in European rabbits [5,6], and endogenous bornaviruses, filoviruses and parvoviruses have been described in mammalian genomes [7,8]. Calicivirus associated with hemorrhagic diseases in European rabbits and hares (*Lepus europaeus* and *Lepus timidus*) have also been described [9].

The origin of the Hepatitis C virus (HCV) remains unclear since its discovery, largely because a closely related animal homolog virus was not identified, until a recently description of a canine homolog of HCV [10]. Unlike most RNA viruses which usually cause acute diseases, HCV establishes life-long, persistent, intrahepatic infections in the majority of the infected individuals, frequently leading to the development of cirrhosis and hepatocellular carcinoma and affects about 170 million people worldwide [11,12]. HCV is a positive single-stranded RNA virus, so called because upon infection their RNA can be directly translated into protein by the host machinery, with an envelope that belongs to the *Flaviviridæ* family [11]. Its genome, of ~9.6 kb, contains one large open reading frame that encodes one large polyprotein that is processed by viral and cellular proteinases to produce the virion structural proteins (core and envelope glycoprotein's E1 and E2) as well as non-structural (NS) proteins (P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B) that form the replication complex [11]. NS3 to NS5B proteins are necessary and sufficient to establish membrane-bound replication complexes that catalyze RNA replication and NS5B protein codes RNA-dependent RNA polymerase (RdRp), because it has a Gly-Asp-Asp (GDD) motif which serves to replicate the HCV-RNA genome [11,13,14].

Chronic HCV infection is currently treated with a combination of pegylated interferon-α and ribavirin but is not always efficient [15]. There are six main genetic types of hepatitis C virus and more than 80 subtypes [16]. The development of antiviral drugs and the molecular studies of HCV have been hampered by a lack of a reliable cell culture system like hepatoma cell lines, African green monkey Vero cells, mosquito cells allowing a persistent *in vitro* virus replication and viral adaptation to the culture [17–20]. To evaluate virus replication in cell cultures, immunofluorescence assay (IFA) and immunogold electron microscopy (IEM) methods among others, were described in previous studies [21,22].

The European rabbit, native to the Iberian Peninsula, is the single recognized progenitor of domestic rabbits [23]. Rabbits have many hereditary diseases common to humans like aortic arteriosclerosis, hypertension, hypertrophic cardiomyopathy, osteoporosis, making them a valuable model in both biomedical and fundamental research [23].

During an investigation addressing the *Flaviviridae* family in the search for significant natural virus's reservoirs of animal diseases we have tested body fluids and liver homogenates samples from domestic, wild rabbits and hare for Bovine Virus Diarrhoea Virus (BVDV) antigen detection using a commercial blocking ELISA (SerelisaTM BVD p80 Ag Mono Indirect, Symbiotics, Lyon - France) and all tested positive. However, when we tested serum and body fluid samples of those animals in the study using an antibody ELISA (SERELISA® BVD p80 Ab Mono Blocking detection kit) that detects specific antibodies to a protein common to all strains of bovine viral diarrhea/mucosal disease (BVD/MD) and border disease (BD) virus (p80/125 non-structural protein) they all tested negative. Efforts to detect significant *Pestivirus* proteins were unsuccessful. These observations and other findings (not shown), led to hypothesize that potential protein cross-reactivity within members of the *Flaviviridae* family could explain the results in that preliminary work.

In this study we were able to detect that homologous DNA fragments coding for HCV core, envelope glycoprotein's E1 and E2, protease NS2–3, serine protease NS3, NS4A, NS5A and the NS5B specific proteins were endogenous in the European rabbit and *Lepus europaeus* genomes. To test this hypothesis, genomic fragments, of structural and NS proteins were tested by RT-PCR, PCR, one dimension gel electrophoresis (1-DE), MALDI-TOF/TOF mass spectrometry including MS/MS peptide sequencing. Blastn with HCV 1b D90208 (HCV database), *Oryctolagus cuniculus* (*O. cuniculus*) and *Homo sapiens* (*H. sapiens*) genomes in Ensembl website (www.ensembl.org) was conducted to evaluate homologies between the virus and the referred species. Moreover, blastn in the same website between *O. cuniculus* and primers used in this study was also performed. To validate the unexpected findings, and to test the biological significance of the HCV homologue detected fragments, suspensions of liver homogenates in this study were inoculated in Mardin-Darby Bovine Kidney (MDBK) cell line and bovine testis (BT) primary cell cultures. HCV fragment homologues replication was detected by IFA and IEM with MAbs for NS3, NS4A and NS5 HCV specific proteins.

## Results

### RT-PCR, PCR and Sequencing of Rabbit and Hare

To investigate if HCV genomic fragments are present in the European rabbit and *Lepus europaeus* genomes we performed RT-PCR and PCR using extracted RNA and DNA respectively from liver homogenates and sequenced all amplified products obtained with these methods. Core, E1/E2, NS5A/B and NS5B HCV genomic proteins were successfully amplified from all samples in this study (Figure S1. A–H), when specific primers were used in both PCRs as previously described [24–28]. RT-PCR from NS5A/B HCV proteins was also performed in WBC and serum samples from 5 domestic rabbits and in body fluids from 6 wild rabbits and one hare (Figure S1. I–J). Amplified fragments were detected only from WBC. Sequencing of RT-PCR and PCR amplified regions was performed.

Alignments and tblastn searches were conducted as a query in the HCV database and whole-genome shotgun in rabbit genome resources at GenBank, NCBI. Retrieved fragment sequences from core, E1/E2, NS5A/B and NS5B HCV specific proteins with 80 to 100% of nucleotides (nt) identities and blast E-values of 8E-09 to 2.4 were identified (Table 1). HCV blast results of selected sequences and regions can be seen in the supporting information, presenting all blasted identities and genotype variability for the core (Table S1), E1/E2 (Table S2) and NS5B (Table S3) proteins.

When blastn of HCV 1b D90208 (HCV database) complete sequence to *O. cuniculus* and to *H. sapiens* genomes was performed in the Ensembl database, match of HCV homolog fragments were detected only for *O. cuniculus* (Table S4). However, blastn against all independent HCV regions to both species showed match of virus homolog fragments for all HCV regions except NS2 and NS5B for *O. cuniculus* and only the HCV 3 untranslated region for *H. sapiens* (Table S4). When blastn of all PCR primers used in this study were performed in Ensembl website, they showed a match with *O. cuniculus* except for HVR1F, Pr3 and Pr4 from E1/E2 and RdRp-NS5B HCV specific regions respectively, as they are degenerated primers (Table S6). Detected homology for both blastn performed showed high percentage of nt identities but was not statistically validated as high E-values were obtained. However, virtually all identical short alignments have relatively high E-values because the calculation of the E-values takes into account the length of the query sequence and shorter sequences have a higher probability of occurring in the database purely by chance [29].

These results suggest some genetic correlations between HCV and *O.cuniculus* and that the matched small HCV homolog fragments are present as repeated sequences throughout *O. cuniculus* genome as identified in PCRs and mass spectrometry results.

### Analysis of Rabbit and Hare by MALDI-TOF/TOF

Liver homogenate proteins were separated by SDS-PAGE and stained with Coomassie Blue. The visible protein bands that were selected on the basis of their molecular weight and that could harbor proteins matching the core, envelope glycoprotein's E1 and E2, serine protease NS3, NS4B, NS5A and NS5B HCV genomic proteins, were excised from the gel, and analyzed by MALDI-TOF/TOF-MS/MS as described by Pinho *et al.* [30]. For each digested sample, the MS and MS/MS spectra were combined for the MASCOT database search. With the matched MS/MS spectra of the European rabbit and *Lepus europaeus* to HCV sequences deposited at Swiss-Prot/UniProt protein database a total of 34 peptide sequences were identified, such as core, envelope glycoprotein's E1 and E2, protease NS2–3, serine protease NS3, NS5A and RdRp-NS5B HCV specific proteins (Table 2). Some MS/MS spectra were retrieved with confidence interval (C.I.)<95%, however they were validated by the associated detected match mass error (−0.08 to 0.09 Da). Among these, for domestic rabbit we were able to identify 16 peptide sequences that match core, envelope glycoprotein's E1 and E2, protease NS2–3, serine protease NS3, NS5A and RdRp-NS5B HCV specific proteins with a C.I. of 71 to 99%, E-value 0.013 to 1.1 and match mass error of −0.08 to 0.09 Da (Table 2, S5). In wild rabbits, 12 peptide sequences that match to the core, serine protease NS3, NS5A and RdRp-NS5B HCV specific proteins were identified with a C.I. of 22 to 93%, E-value 0.016 to 1.1 and match mass error of −0.08 to 0.06 Da (Table 2, S5). Lastly, from hare we were able to identify 6 peptide sequences that match core, envelope glycoprotein E2, protease NS2–3, NS5A and RdRp-NS5B HCV specific proteins with a C.I. of 53 to 82%, E-value 0.067 to 0.92 and match mass error of −0.07 to 0.04 Da (Table 2, S5). Rabbit liver samples were fresher than the corresponding from the hare sample, which could explain the less number of sequence identities detected in the latter specie. Spectra generated by MS/MS analysis from all identified peptide sequences can be observed in Figure S2. NS4B protein could not be identified by MS/MS analysis in our study.

The most representative HCV genotype that matches with the identified peptide sequences is 1b. Furthermore, when we matched all identified peptide sequences with the Swiss-Prot accession number POLG_HCVJA (genotype 1b), a total genome poly-

HCV Homolog Fragments in Leporidae Genomes

**Table 1.** Endogenous HCV fragment sequences generated by PCR and RT-PCR from the European rabbit *and Lepus europaeus* liver samples.

| HCV region | Primers | Identified nucleotides | Acession no. | Genotype | Position | E-value | % Identities |
|---|---|---|---|---|---|---|---|
| Core | 104/134 | GTGACCGCTCGGAAGTCTTCC[a] | GU441256 | 1b | 304-284* | 0.005 | 100 |
| Core | 104 | CGAGGTTCCCGTCCCTCTTGG | U10230 | 2a | 301–321 | 0.33 | 100 |
| Core | 134 | CGACCGCTCGGAAGTCTTCCTA | HM049503 | 2a | 508-487 | 0.004 | 100 |
| E1/E2 | HVR1F/R | TTTGGAAAAGGCCAGGGAA | AM885177 | 1a | 232-214* | 0.37 | 94 |
| E1/E2 | HVR1R | TTTTGTTTCCGAAGCCAATACATCCA[b] | AY767496 | 3a | 133–158 | 0.12 | 84 |
| E1 | HVR1F | CCACCAAAACCGTCCCTGGCCTTTTCCAAA | AM885166 | 1a | 203–232 | 2.4 | 80 |
| NS5B | AS/S | GCTGGGGACTTGTGCCTTCAGGGGACATGTGG | EF116149 | 6a | 235-204* | 0.014 | 81 |
| NS5B | S | AGCCTCCGTGAAAGCTTG | HM009084 | 1b | 303-286* | 1.1 | 94 |
| NS5B | S | CAAGCTTTCACGGAGGCTATGACCAGGAATTCCGCC | AY685047 | 4p | 366–401* | 4e-08 | 100 |
| NS5B | AS | AATCAAAGCAGCGGGTATCATACGGGATCCCCA | AY743071 | 4p | 33-1* | 8e-07 | 100 |
| NS5B | Ṡ | TCTTCACGGAGGCTATGACTAGGTATTCTGCC | GU589872 | 1 | 348–379 | 2e-04 | 93 |
| NS5B | AṠ | GATCCCGTATGATACCCGCTGCTTTGA | DQ508484 | 1b | 5–31 | 6e–06 | 100 |
| NS5B | AṠ | TCAAAGCAGCGGGTATCATACGGGATCCCCA | DQ345619 | 1b | 32-2* | 6e-05 | 100 |
| NS5B | Pr3/Pr4 | GCTGGGGACTTGTGCCTTCAGGGGACATGTGG | EF116149 | 6a | 235-204* | 0.014 | 81 |
| NS5B | Pr3/4 | AGCTTTCACGGAGGCTATGACCAGGTACTCAGC[c] | DQ238690 | 2b | 66–98 | 8e-09 | 100 |
| NS5B | Pr3/4 | AGCTTTCACGGAGGCTATGACCAGATATTCAGCA | DQ663603 | 3a | 327–360 | 4e-07 | 100 |
| NS5B | Pr4 | TATGATACCCGCTTGCTTTGA | X88622 | 1a | 14–29 | 1.1 | 100 |
| NS5B | Pr4 | GTAGAGTCGAAGCAACGGGTAACATA | EU255955 | 1a | 8190-8165 | 0.023 | 96 |

Nucleotide homology between the studied samples and HCV sequences deposited at the site (http://hcv.lanl.gov/content/sequence/BASIC_BLAST/basic_blast.html) are labeled in bold and underlined. See Supplementary Information for whole blast,
[a]Table S1.,
[b]Table S2,
[c]Table S3.
*Reverse complement.
doi:10.1371/journal.pone.0049820.t001

protein coverage of 12% (351/3010 aa) (Figure S3) was obtained. It is important to note that these match cover 28% (53/190 aa) of the core, 6% (11/192 aa) of the E1, 9% (34/363 aa) of the E2, 22% (47/217 aa) of the NS2–3, 12% (73/631aa) of the NS3, 17% (74/447 aa) of the NS5A and 10% (59/591 aa) of the RdRp-NS5B structural and NS HCV 1b genome polyprotein.

### IFA and IEM of Rabbit and Hare in Bovine Cell Cultures

To address the biological significance of these findings and of the question whether these HCV homolog fragments can replicate in cell culture, MDBK cell line and primary BT cell cultures were inoculated with suspensions of liver homogenate samples from the wild, domestic rabbits and the hare. Inoculated cells were harvested at the second subculture (14 days post infection) and tested by IFA using mouse MAbs anti-HCV NS3, anti-HCV NS4A and anti-HCV NS5, specific immunostaining for the three selected antibodies could be demonstrated (Figure 1). As MDBK cell line showed a better signal for European rabbit and *Lepus europaeus* endogenous HCV homolog fragments replication than BT primary cell cultures by IFA, they were selected for treatment by IEM (Figure 2). Negative controls (uninoculated MDBK and BT cells) were included and treated with the selected MAbs and no reaction was detected (Figure 1, 2).

## Discussion

Hepatitis C virus, whose origin remains unclear, is the major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma in humans [11]. The HCV diagnosis in humans is usually done using serum or plasma of infected patients to detect virus or specific antibodies in circulation [31]. The absence of amplicons from serum and body fluids in the present study is an indication that viral particles were not in circulation. On the other hand, all positive samples were originated from samples containing only cells, suggesting that the detected virus fragments are endogenous in the European rabbit and *Lepus europaeus* genomes.

Endogenous *flaviviruses* have previously been reported in the genomes of *A. aegyptii* and *A. albopictus* mosquitoes [32,33] and complete putative genomic structures have not been determined. Moreover, recently *Flavivirus* were also detected in mosquito species using degenerated primers designed for a *Flavivirus* generic RT-nested-PCR assay [34,35] what is in agreement with our results, when testing rabbits and hare samples by PCR and RT-PCR using HCV specific primers. Endogenous viral elements have also been identified and demonstrated in animal genomes, and it has been suggested that its diverse sequences may account for the gene flow [36] between animals and viruses.

The sequenced fragments in our study were homologous to various HCV types, and included regions that are commonly used in HCV research or diagnosis (Table 1). The detected fragments belong to conserved regions in the virus, which are common to several types and/or subtypes (Table 1, S1, S2, S3). These findings demonstrate that these fragments are endogenous in the European rabbit and *Lepus europaeus* genomes with a high probability to appear as repeated sequences throughout their genome as identified when HCV 1b D90208 and HCV specific primers blastn were performed in the Ensembl website against the *O. cuniculus* genome (Table S4, S6). The findings could also suggest

**Table 2.** HCV homolog proteins identified by MALDI-TOF/TOF-MS/MS analysis from the European rabbit and *Lepus europaeus* liver homogenates.

| Animal | Accession no.† (genotype) | Protein name | Best peptide sequence | Genome position (aa) | Match error (Da) | Total ion score C I % | E-value |
|---|---|---|---|---|---|---|---|
| DR | POLG_HCVJA (1b) | Core | STNPKPQR | 2–9 | −0.02 | 99 | 0.39 |
| DR | POLG_HCVJA (1b) | Core | SQPRGRR | 56–62 | −0.02 | 99 | 0.23 |
| DR | POLG_HCVJA (1b) | Core | TWAQPGYPWPLYGNEGMGWAGWLLSPR | 75–101 | 0.07 | 99 | 0.3 |
| DR | POLG_HCVJA (1b) | E1 | NSSIPTTTIRR | 250–260 | −0.04 | 99 | 0.022 |
| DR | POLG_HCVJA (1b) | E2 | VASSTQSLVSWLSQGPSQK | 392–410 | −0.07 | 98 | 0.046 |
| DR | POLG_HCVVA (2K) | E2 | SIEEFR | 461–466 | 0.03 | 71 | 0.58 |
| DR | POLG_HCVBK (1b) | NS2–3 | KVAGGHYVQMAFMK** | 927–940 | −0.04 | 95 | 0.047 |
| DR | POLG_HCVJA (1b) | NS3 | GPITQMYTNVDQDLVGWPAPPGAR | 1095–1118 | 0.06 | 81 | 0.6 |
| DR | POLG_HCVJ1 (1b) | NS3 | AVDFIPVESLETTMR | 1192–1206 | −0.07 | 87 | 0.049 |
| DR | POLG_HCVJA (1b) | NS5A | DVWDWICTVLSDFKTWLQSKLLPR* | 1979–2002 | 0.09 | 81 | 0.013 |
| DR | POLG_HCVT5 (6b) | NS5A | IPGIPFISCQAGYR* | 2008–2021 | −0.01 | 89 | 0.15 |
| DR | POLG_HCVK3 (3a) | NS5A | NGSMRLAGPR** | 2047–2056 | −0.02 | 74 | 0.92 |
| DR | POLG_HCVSA (5a) | NS5A | GSPPSLASSSASQLSAPSLK | 2194–2213 | −0.08 | 90 | 0.087 |
| DR | POLG_HCVJA (1b) | NS5B | VEFLVNTWK | 2620–2628 | 0.01 | 81 | 0.15 |
| DR | POLG_HCVSA (5a) | NS5B | AAIRSLTQR | 2674–2682 | −0.02 | 99 | 0.1 |
| DR | POLG_HCVJA (1b) | NS5B | AFTEAMTR | 2757–2764 | −0.01 | 81 | 1.1 |
| WR | POLG_HCVNZ (3a) | Core | SQPRGRR | 56–62 | 0.04 | 22 | 1.1 |
| WR | POLG_HCV6A (6a) | NS3 | CDELAGKLKSLGLNAVAFYR* | 1405–1424 | −0,04 | 87 | 0.15 |
| WR | POLG_HCVJ8 (2b) | NS3 | GRLGVYR | 1498–1504 | −0.04 | 57 | 0.05 |
| WR | POLG_HCVJF (2a) | NS3 | AKAPPPSWDAMWKCLAR* | 1601–1617 | 0.04 | 93 | 0.016 |
| WR | POLG_HCVVO (6K) | NS5A | NGSMRISGSR | 2043–2052 | 0.04 | 22 | 0.05 |
| WR | POLG_HCVVN (6d) | NS5A | IVGPKMCSNVWNNR* | 2044–2057 | 0.01 | 87 | 0.2 |
| WR | POLG_HCVCO (1b) | NS5A | VGDFHYVTGMTTDNVK** | 2096–2111 | 0.06 | 74 | 0.5 |
| WR | POLG_HCVCO (1b) | NS5A | GSPPSLASSSASQLSAPSLK | 2193–2212 | −0.08 | 74 | 0.53 |
| WR | POLG_HCVJ6 (2a) | NS5A | SDLEPSIPSEYMLPKKR | 2264–2280 | −0.05 | 22 | 0.1 |
| WR | POLG_HCV6A (6a) | NS5B | SASLRQK | 2472–2478 | 0.00 | 87 | 0.13 |
| WR | POLG_HCVJ8 (2b) | NS5B | LLTVEEACALTPPHSAK* | 2524–2540 | −0,05 | 57 | 0.15 |
| WR | POLG_HCV6A (6a) | NS5B | MALYDVTR** | 2601–2608 | 0.01 | 87 | 0.2 |
| Hare | POLG_HCVJP (2b) | Core | GSRPTWGPSDPRHR | 102–115 | 0.04 | 53 | 0.13 |
| Hare | POLG_HCVJP (2b) | E2 | LWHYPCTVNFTIFKVR* | 619–634 | −0.04 | 53 | 0.65 |
| Hare | POLG_HCVJK (3K) | NS2–3 | LGKEVLLGPADDYR | 1011–1024 | −0.07 | 81 | 0.067 |
| Hare | POLG_HCVK3 (3a) | NS5A | NGSMRLAGPR** | 2047–2056 | −0.05 | 67 | 0.92 |
| Hare | POLG_HCVJP (2b) | NS5B | AASKVSAR | 2516–2523 | 0.00 | 53 | 0.65 |
| Hare | POLG_HCVT5 (6b) | NS5B | DVRSHTSK | 2535–2542 | 0.04 | 82 | 0.09 |

DR – Domestic rabbit *(Oryctolagus cuniculus)*, WR – Wild rabbit *(Oryctolagus cuniculus.)*, Hare (*Lepus europaeus*),
†– SwissProt accession number,
*Modification – Carbamidomethyl (C),
**Modification – Oxidation (M).
doi:10.1371/journal.pone.0049820.t002

that the different animals could have equal and different HCV homolog specific peptides in their genomes (Table 2) and therefore, excluding the possibility of contamination between samples. All the identified peptides are located in HCV specific proteins which have several important determinants or functions like for example host/cell tropism, viral immunogenicity and participation in virus replication complex. Furthermore, the obtained results demonstrate that the studied species have some homologous genetic make-up with the members of the *Flaviviridae* family. Miller and Purcell [37] described significant homology between HCV and two *Pestivirus* polyproteins, which is in line with our results.

The analysis of the sequences that were generated either by PCR or MALDI techniques showed a considerable degree of diversity, which is similar to what is observed for HCV in nature. This diversity explains, at least in part, the difficulties involved in the development of effective vaccines and therapies for the disease [38,39]. However, more studies should be performed to evaluate the significance of the peptide sequences distributed along the HCV genome and which are endogenous to the genomes of the studied species.

HCV Homolog Fragments in Leporidae Genomes

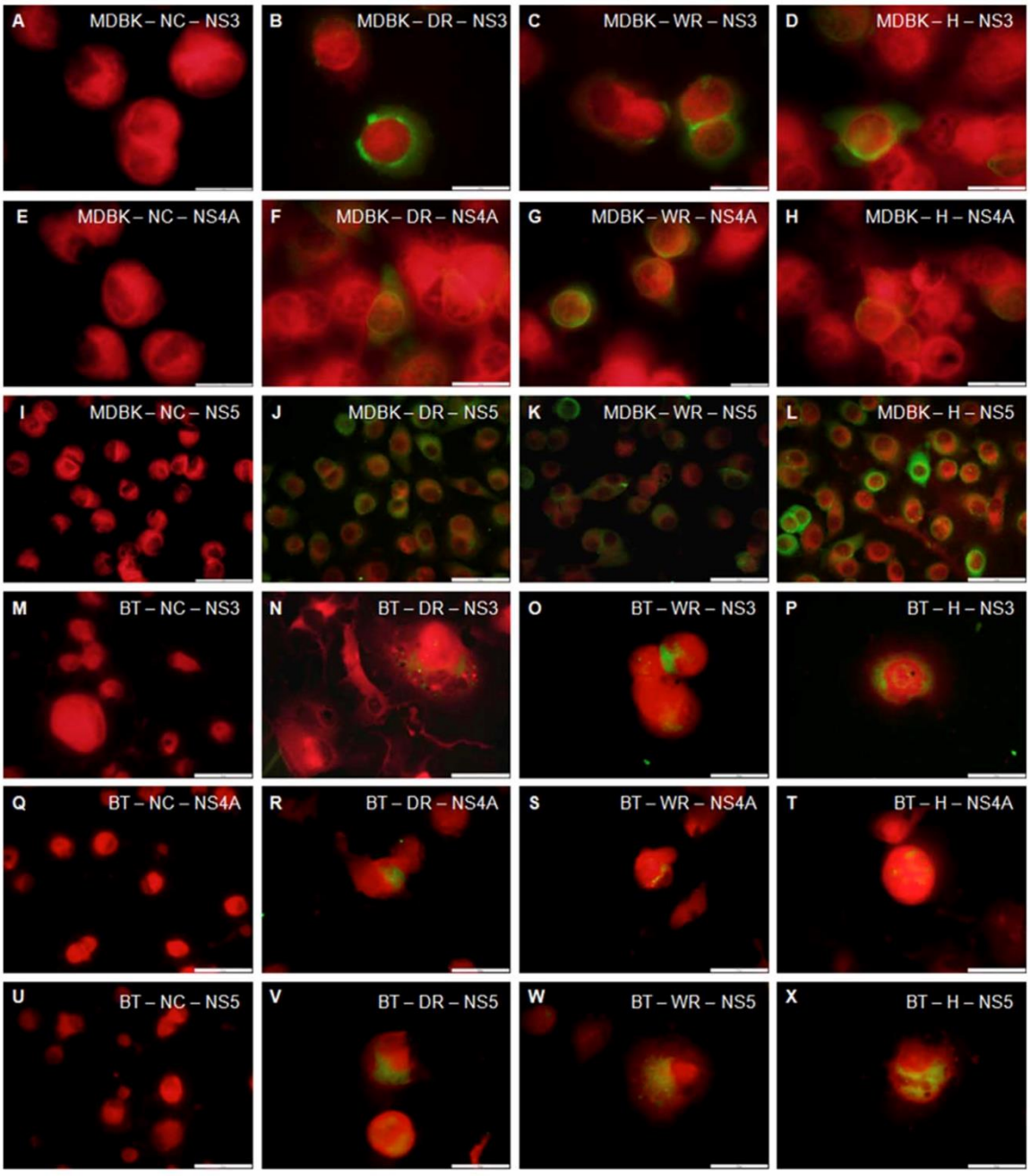


**Figure 1. Detection of HCV proteins by immunofluorescence in MDBK and BT cells.** The cells infected with liver homogenates from domestic (DR), wild (WR) rabbits and a hare (H) were analyzed 14 days post infection. MDBK cells incubated with MAbs specific for NS3 (A to D), NS4A (E to H) and NS5 (I to L) proteins. BT cells incubated with MAbs specific for NS3 (M to P), NS4A (Q to T) and NS5 (U to X) proteins. Negative controls (NC) for each cell culture and each Mab was also performed (A, E, I, M, Q and U). No fluorescence was detected. Scale bars: (A to H), 20 μm; (I to X), 50 μm.
doi:10.1371/journal.pone.0049820.g001

We demonstrate that HCV homologous fragments present in the rabbit and hare genomes can replicate in bovine cell cultures (Figure 1, 2), although no virus-like particles were detected in the liver homogenates of the tested samples. In the cell negative controls no immunofluorescence or immunogold particles were detected. Immunogold particles were detected in different cell

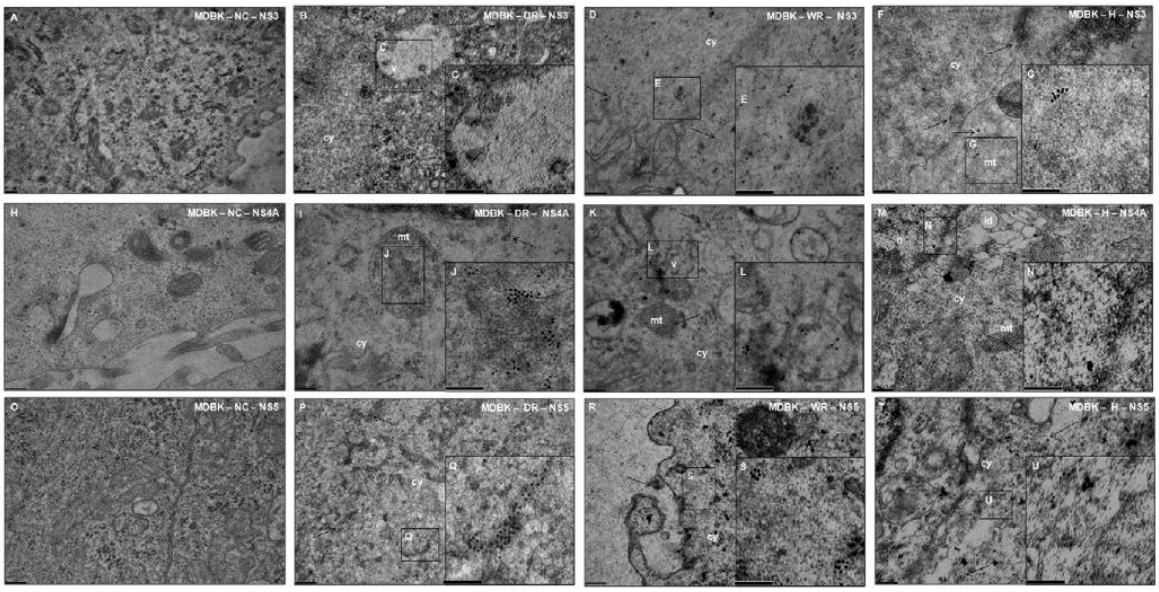


**Figure 2. Immunogold TEM detection of HCV proteins in MDBK cells.** The cells infected with liver homogenates from domestic (DR), wild (WR) rabbits and a hare (H), were analyzed 14 days post infection. The cells were incubated with MAbs specific for NS3 (A to F), NS4A (H to M) and NS5 (O to T) proteins. High-magnification views of immunogold particles in several organelles are shown in boxes (C, E, G, J, L, N, Q, S and U). The immunogold particles were detected in vacuoles (v) (C and L), in mitochondria (mt) (G and J), cytoplasm (cy) (E, Q, S and U) and nucleous (n) (N). Arrows indicate the presence of gold particles detected in the same organelles (mt, cy) and close to the extracellular membrane (R). Negative controls (NC) for each MAb were also performed (A, H and O). No immunogold particles were detected. The immunogold particles were 10 nm in diameter. Scale bars: (A) 0.2 μm; (B, D, F, H, I, K, M, O, P, R and T), 200 nm; (C, E, G, J, L, N, Q, S and U), 100 nm.
doi:10.1371/journal.pone.0049820.g002

organelles such as vacuoles, nucleus, lipid droplets, mitochondria and cytoplasm consistent with others reports previously described for HCV replication in cell cultures [40,41]. These findings may be an insight into new approaches for therapy and molecular studies.

This study provides the evidence of endogenization of HCV homolog fragments in European rabbit and *Lepus europaeus* genomes and demonstrates that the detected fragments are infectious and replicate in bovine cell culture systems. Our findings may lead to novel research approaches on the HCV origin, genesis and diversity. The timeframe of the HCV integration event in the rabbit genome or virus origin are still to be determined and warrants further molecular, biologic and phylogenetic investigations. The biological and epidemiological implications of these findings, which have to be further explored, may also suggest the European rabbit and *Lepus europaeus* as possible candidate models for functional and evolutionary studies.

## Materials and Methods

### Ethics Statement

Animal Care and Use Committee approval was not obtained for this study because bovine testicles were collected from bovine fetuses after the dam's slaughter at an authorized slaughterhouse. Animals were protected at slaughter according to the Council Directive 93/119/EC of 22 December 1993, and bovine testicles collection for experimental purposes was done by qualified members as previously authorized by the Portuguese National Authority for Animal Health (Direcção Geral de Veterinária) of the Ministry for Agriculture, Sea, Environment and Spatial Planning (Ministério da Agricultura, do Mar, do Ambiente e do Ordenamento do Território).

### Samples

Liver homogenates from 6 wild and 6 domestic rabbits and 1 hare, 5 serum and WBC samples of domestic rabbits and 7 samples of body fluids of the 6 wild rabbits and the hare were used in this study. These samples belong to a collection of the Laboratory of Infectious Diseases of the Veterinary Clinics Department at ICBAS, University of Porto [42,43].

### RNA, DNA Extractions and cDNA Synthesis

Total RNA was extracted from 10% (w/v) liver homogenates with TRI® Reagent LS (Sigma-Aldrich, Steinheim, Germany). From the RNA extracts cDNA was synthesized using random hexamers (Amersham, New Jersey, USA). Briefly, 5 μl of RNA was mixed with 2 μl of diethyl pyrocarbonate (Sigma-Aldrich, Steinheim, Germany) water and 1 μl (0.02 U) of random hexamer, denaturated at 65°C for 10 min and then cooled on ice for 10 min. Thereafter, the hexamers were extended for 90 min at 37°C by 1 μl Moloney murine leukaemiavirus reverse transcriptase (200 U) (Invitrogen™, Paisley, UK) [44]. in the presence of 2.0 μl dNTPs (10 mM), 5 μl 1st standard buffer and 1 μl RNAse inhibitor (Roche, Mannheim, Germany). The enzyme was then inactivated for 5 min at 95°C and the cDNA was immediately used or kept at −20°C until further processed. Genomic DNA was extracted from the same samples as RNA using the QIAamp® DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer instructions.

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### RT-PCR, PCR and Sequencing of the Amplified Fragments

Using cDNA and DNA, RT-PCR and PCR were performed targeting the specific HCV structural and NS proteins, core, envelope glycoprotein's E1/E2, NS5A/B and NS5B [24–28], in a thermocycler C 1,000 (Bio-Rad, California, USA). Briefly, the PCR mix (25 µl) contained 2.5 µl of 10× PCR buffer, 0.6 µl dNTPs (10 mM), 10 pmol of each primer, 2.5 µl $MgCl_2$ (25 mM) and 1 U Taq DNA polymerase (Fermentas, St. Leon-Rot, Germany). PCR cycling conditions were performed as described above with the following primers: sense no. 256 (5′ CGCGCGACTAGGAAGACTTC 3′) and antisense no. 186 (5′ ATGTACCCCATGAGGTCGGC 3′) for the core PCRI; sense no. 104 (5′ AGGAAGACTTCCGAGCGGTC 3′) and antisense no. 134 (5′ CCAAGAGGGACGGGAACCTC 3′) for the core PCRII; sense HVR1F (5′ TGCTGGGTCCARRTYACCCC 3′) and antisense HVR1R (5′ GCTGTCATTACAGTTAAGGGCA 3′) for the E1/E2 (HVR-1); sense S (5′ TGGGGATCCCGTATGATACCCGCTGCTTTGA 3′) and antisense AS (5′ GGCGGAATTCCTGGTCATAGCCTCCGTGAA 3′) for the NS5A/B PCRI; sense S′ (5′ TGCGGTTATTGCCGTTGTCGCGCCAGCGG 3′) and antisense AS′ (5′ GGCAGAATACCTAGTCATGGCCTCTGTGAA 3′) for the NS5A/B PCRII; sense Pr3 (5′ TATGAYACCCGCTGYTTTGACTC 3′) and antisense Pr4 (5′ GCNGARTAYCTVGTCATAGCCTC 3′) for the RdRp-NS5B. The amplified products were analyzed on a 1.2% (w/v) agarose gel stained with gel red™ (Biotium, California, USA) and visualized under UV light (Bio-Rad, California, USA). The amplicons were directly sequenced at STAB Vida (C. Caparica, Portugal) after being purified using a Qiagen kit (Qiagen, QIAquick® Gel Extraction Kit, Crawley, UK) using the same primers.

### Proteins Extraction and Quantification

Proteins were extracted from 10% (w/v) liver homogenates following the Alliance for Cellular Signaling (AFCS) protocol [45]. Protein concentration was determined in a Qubit® Fluorometer (Invitrogen, Carlsbad, USA) according to the manufacturer instructions.

### Proteomic Analysis by 1-DE and MALDI-TOF/TOF-MS/MS

SDS-PAGE, 1-DE, was performed by Laemmli method [46] After extraction, proteins were separated by 12% SDS-PAGE using 30 µg of total protein in a Mini-Protean® 3 Cell (Bio-Rad, California, USA) system. After SDS-PAGE separation, proteins were Coomassie blue stained (Imperial Protein stain, Thermo Scientific, Rockford, USA).The protein bands of interest were reduced, alkylated and *in gel* digested with trypsin accordingly to trypsin manufacturer's suggested protocol (Promega, USA). The resulting peptides were concentrated with ZipTips (Millipore, USA) in agreement with manufacturer's instructions and eluted into the MALDI plate using alpha-cyano 4-hydroxycinnamic acid as a matrix as already described [30]. MS and MS/MS peptide mass spectra were acquired with a MALDI-TOF/TOF 4700 Proteomics Analyzer (ABSCIEX, USA). Peptide mass spectra were obtained in reflector positive mode for a mass window of 700–4000 Da. Some peptides were selected for MS/MS fragmentation by collision induced dissociation (CID) in MS/MS positive mode. Proteins were identified using the combined information of MS and MS/MS spectra by the GPS (Global Proteome Server) Explorer Software v3.6 (ABSCIEX, USA) which integrates the Mascot protein search engine v2.1.04 (Matrix Science, UK) configured to perform searches at the Swiss-Prot/UnitProt database. This procedure was performed at the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP) Proteomics Unit.

### Cell Culture and Sample Inoculation

MDBK cells (American Type Culture Collection) [47] obtained from the Friedrich-Loeffler-Institute (Insel Riems, Germany) were maintained at 37°C, 5% $CO_2$ in D-MEM (Invitrogen™, Paisley, UK) supplemented with 10% heat-inactivated horse serum (Invitrogen™, Paisley, UK), penicillin (100 U/ml) – streptomycin (100 µg/ml) (Sigma-Aldrich, Steinheim, Germany) and sodium pyruvate (1 mM) (Invitrogen™, Paisley, UK). Primary BT cells were obtained from bovine testis collected in a local cattle slaughterhouse (Famalicão, Portugal) using methods previously reported [48] with some modifications. Briefly, testicles from bovine fetuses recovered at an abattoir from pregnant cows, were excised and the capsule was removed. The tissue was chopped with sterile scissors into pieces approximately 1 $mm^3$ and washed with phosphate buffered saline (PBS) until rinse water is clear. The chopped tissue was added to a flask with solution of 0.25% trypsin (Sigma-Aldrich, Steinheim, Germany) at 37°C, a magnetic stirring bar and mixed at low speed for 10 min. New 0.25% trypsin solutions were added until large digested tissue fragments settle in the flask. Final digest was filtered through three layers sterile gauze to an iced flask for 3 times and centrifuged for 10 min at 6000xg. Supernatant was discarded and cells were resuspended in D-MEM (Invitrogen™, Paisley, UK) supplemented with 10% FBS (Invitrogen™, Paisley, UK), penicillin (100 U/ml) – streptomycin (100 µg/ml) (Sigma-Aldrich, Steinheim, Germany) at 37°C, 5% $CO_2$. BT cells were maintained in the same conditions or stored in liquid nitrogen. Suspensions of liver homogenate samples (1 ml) filtered at 0.2 µm (Sarstedt, Nümbrecht, Germany) were inoculated on both cell cultures and these were subcultured once a week up to 7 and 2 passages of MDBK and BT cultures respectively. Uninoculated MDBK and BT cell cultures were also maintained in all passages as negative controls for all procedures.

### Immunofluorescence Assay

Immunofluorescence evaluation of HCV NS3, NS4A and NS5 specific proteins in MDBK and BT cells was performed as previously described [21], with some modifications. Cells inoculated with liver homogenate samples and the negative controls (uninoculated MDBK and BT cells) were subcultured 14 days post inoculation (second passage) and a drop of cell suspensions was placed on glass slides dots (6 mm) (Poly Labo, Strasbourg, France), pretreated with poly-L-lysine solution (Sigma-Aldrich, Steinheim, Germany). After incubation during 4 hours at 37°C, 5% $CO_2$, the slides were fixed with ice-cold acetone (Merck, Darmstadt, Germany) and then treated with a blocking solution for 30 min. The slides were then incubated with mouse MAbs anti-HCV NS3, anti-HCV NS4A and anti-HCV NS5 (all from Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:100) in blocking solution at room temperature for 60 min, and subsequently incubated with goat anti-mouse IgG-FITC (Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:200) for 30 min. The slides were then counterstained with Evans blue (Sigma-Aldrich, Steinheim, Germany) for 5 min, washed 3 times with 1x PBS, mounted with florescence mounting solution and visualized in a fluorescence microscope (BX51, equipped with a DP72 camera and software Cell-B, Olympus, Tokyo, Japan).

### Immunogold Electron Microscopy

For IEM, 20 µl of cell suspensions at 14 days post inoculation from each MDBK cell flasks with the liver samples and the negative controls (uninoculated cells) were processed according to

procedures previously described [22], with some modifications. In brief, cells were fixed in 1.25% glutaraldehyde (Electron Microscopy sciences, Hatfield, USA) and 2% paraformaldehyde (Merck, Darmstadt, Germany) in Tris Buffered Saline (TBS), dehydrated and embedded in Epon resin (TAAB, Berks, England). Ultrathin sections cut and placed on nickel grids (TAAB, Berks, England) and were floated for 10 min on a drop of TBS in a moist chamber. The grids were then allowed to float for 30 min on a drop of 14.4% sodium metaperiodate (Sigma-Aldrich, Steinheim, Germany) and then washed with TBS. After blocking with 2% BSA (Sigma-Aldrich, Steinheim, Germany) in TBS the grids were incubated for 60 min on a drop of the same primary MAbs used in IFA procedure diluted 1:50 in TBS-BSA overnight at 4°C and then washed for several times. Then grids were incubated for 60 min in a drop of secondary anti-mouse IgG gold antibody (Sigma-Aldrich, Steinheim, Germany) diluted 1:20 in TBS-BSA and then washed with TBS. Grids were counterstained with uranyl acetate and lead citrate as Reynolds [49] methods and visualized in a transmission electron microscope operated at 60 kV by Jeol 1400 (Tokyo, Japan) with a CCD digital camera Orious 1100W Tokyo, Japan at the Institute for Molecular and Cell Biology (IBMC) of the University of Porto, HEMS.

## Supporting Information

**Figure S1 PCR (A, C, E, and G) and RT-PCR (B, D, F, and H) generated amplicons of the HCV genomic regions from liver samples.** (A, B) Core protein; (C, D) Envelope proteins E1/E2; (E, F) NS5 A/B proteins and (G, H) RdRp-NS5B protein in domestic (1DR to 6DR); wild (1WR to 6WR) rabbits and hare (H). RT-PCR (I, J) generated amplicons of NS5 A/B protein in domestic rabbit serum (1DRS to 5DRS), domestic rabbit buffy coat (1DRB to 5DRB), wild rabbit's body fluids (1WRF to 6WRF) and hare body fluids (HF). Negative control (NC); Marker 5 (Eurogentec, Seraing, Belgium) (M).
(TIF)

**Figure S2 MALDI-TOF/TOF-MS/MS spectra of the peptide sequences identified with HCV homolog fragments on liver samples.** Domestic rabbits (1–16), wild rabbits (17–28) and hare (29–34). Da – Dalton; C. I. – confidence interval; STNPKPQR (1), SQPRGRR (2), TWAQPGYPWPLYGNEGMGWAGWLLSPR (3), NSSIPTTTIRR (4), VASSTQSLVSWLSQGPSQK (5), SIEEFR (6), KVAGGHYVQMAFMK (7), GPITQMYTNVDQDLVGWPAPPGAR (8), AVDFIPVESLETTMR (9), DVWDWICTVLSDFKTWLQSKLLPR (10), IPGIPFISCQAGYR (11), NGSMRLAGPR (12), GSPPSLASSSASQLSAPSLK (13), VEFLVNTWK (14), AAIRSLTQR (15), AFTEAMTR (16), SQPRGRR (17), CDELAGKLKSLGLNAVAFYR (18), GRLGVYR (19), AKAPPPSWDAMWKCLAR (20), NGSMRISGSR (21), IVGPKMCSNVWNNR (22), VGDFHYVTGMTTDNVK (23), GSPPSLASSSASQLSAPSLK (24), SDLEPSIPSEYMLPKKR (25), SASLRQK (26), LLTVEEACALTPPHSAK (27), MALYDVTR (28), GSRPTWGPSDPRHR (29), LWHYPCTVNFTIFKVR (30), LGKEVLLGPADDYR (31), NGSMRLAGPR (32), AASKVSAR (33), DVRSHTSK (34). For more detailed information refer to Table 2.
(DOC)

**Figure S3 MALDI-TOF/TOF-MS/MS analysis of European rabbit and *Lepus europaeus* liver homogenates.** The peptides that match core, envelope glycoprotein's E1and E2, protease NS2–3, serine protease NS3, NS5A, RdRp-NS5B HCV structural and non-structural proteins, with the reference strain HCV genotype 1b (Swiss-Prot/UniProt accession number POLG_HCVJA), are shown in boldface and underlined. Peptide sequences cover 12% (351/3010 aa) of the total genome polyprotein of the selected reference strain. Core (A) (positions 2–19), the matched peptides cover 28% (53/190 aa) of the protein. E1 (B) (positions 192–383), the matched peptides cover 6% (11/192 aa) of the protein. E2 (C) (positions 384–746), the matched peptides cover 9% (34/363 aa) of the protein. NS2–3 (D) (positions 810–1026), the matched peptides cover 22% (47/217 aa) of the protein. NS3 (E) (positions 1027–1657), the matched peptides cover 12% (73/631 aa) of the protein. NS5A (F) (positions 1973–2419), the matched peptides cover 17% (74/447 aa) of the protein. NS5B (G) (positions 2420–3010), the matched peptides cover 10% (59/591 aa) of the protein.
(TIFF)

**Table S1** Homolog core HCV genomic fragment present in the European rabbit and *Lepus europaeus* genomes.
(DOC)

**Table S2** Homolog E1/E2 HCV genomic fragment present in the European rabbit and *Lepus europaeus* genomes.
(DOC)

**Table S3** Homolog NS5B HCV genomic fragment present in the European rabbit and *Lepus europaeus* genomes.
(DOC)

**Table S4** Blastn between HCV 1b, *O. cuniclus* and *H. sapiens*.
(DOC)

**Table S5** All HCV homolog proteins identified by MALDI-TOF/TOF-MS/MS analysis from the European rabbit and *Lepus europaeus* liver homogenates.
(DOC)

**Table S6** Blastn between HCV specific primers and *O. cuniclus*.
(DOC)

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## Author Contributions

Conceived and designed the experiments: GT ES. Performed the experiments: ES HO. Analyzed the data: GT ES SM HO JC. Wrote the paper: ES GT SM HO JC.

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## 3.3 - Hepatitis C-like Viruses are Produced in Cells from Rabbit and Hare DNA

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# Hepatitis C-like viruses are produced in cells from rabbit and hare DNA

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**Hepatitis C virus (HCV), a major causative agent of acute and chronic liver disease, belongs to the *Flaviviridæ* family and contains a single-strand positive-sense RNA genome, which upon virus entry and uncoating, functions as mRNAs and thus can be directly translated into proteins by host cell machinery. To date the HCV origin remains unclear and HCV life cycle and pathogenesis are not enlightened processes due to the absence of HCV efficient cell cultures systems or animals models. Here we show that rabbit and hare HCV-like viruses, RHCV and HHCV respectively, are formed after the inoculation of genomic DNA in Madin-Darby bovine kidney cell line cultures. RHCV is closely related to the HCV-1a/HCV-1b genotypes and HHCV is more closely related to the HCV-1b genotype. These findings could contribute to the understanding of HCV origin as well as clarify the virus life cycle, pathogenesis, evolution and diversity.**

Positive RNA viruses are characterized by a single-strand positive-sense RNA (ssRNA+) genome, which, upon virus entry and uncoating, functions as mRNAs and thus can be directly translated into proteins by host cell machinery[1]. Therefore, the genomic RNA could work as template for viral RNA replication. Following translation and processing of the viral polyprotein(s), viral nonstructural (NS) proteins, viral RNA, and host factors form membrane-associated replication complexes that carry out viral RNA synthesis[2,3]. The resultant progeny positive RNA strands can either initiate a new translation cycle or be packaged into virions that are subsequently released to infect naïve cells[2]. Moreover, host factors participate in almost all or probably in all steps of ssRNA+ virus infection, including entry, viral gene expression, virion assembly and release[2]. Furthermore, host factors are targeted by ssRNA+ viruses to modulate host gene expression and defenses[2].

Studying the presence and behavior of endogenous viral elements (EVEs) having ancestries with ssRNA+ viruses in species genomes is another interesting challenge because all genetic processes involving them are poorly understood. Most RNA virus EVEs identified to date are derived from RNA viruses with negative-sense genomes or from RNA viruses that lack DNA intermediates[4–6]. EVEs derived from flavivirus-related RNA viruses were first described in insects in 2004[7]. This description involves a gene sequence of an RNA virus that replicate using an RNA-dependent RNA polymerase integrated into the Aedes spp. mosquitoes genome[7]. Recently, Katzourakis and Gifford described the presence of EVEs derived from RNA viruses, ssRNA+viruses included, in animal genomes and this has revolutionized the understanding of the processes and time-scale of viral evolution[8]. More recently, EVEs that share a sequence similarity to ssRNA+ viruses of plants integrated into the genomes of insects have also been

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reported[9]. Although, EVEs derived from ssRNA+ viruses appearing in extremely low copy numbers in the genomes; one genomic copy in the case of the *Reoviridae*, five in the case of the *Flaviviridae* and a small number in Potato virus Y were previously described[10].

Hepatitis C virus (HCV), a major causative agent of acute and chronic liver disease, belongs to the *Flaviviridæ* family and contains an ssRNA+ genome of ~9,600 nucleotides that encodes a polyprotein precursor of ~3,000 amino acids[11]. Consequently and as described previously, probably the ssRNA+ genome could function as mRNAs that can be directly translated into proteins by host cell machinery and genomic RNA could function as template for viral RNA replication.

We recently described the presence of endogenous HCV homolog fragments in wild/domestic rabbits (*Oryctolagus cuniculus*) and hare (*Lepus europaeus*) genomes and the capacity of these small endogenous fragments to replicate in Mardin-Darby Bovine Kidney (MDBK) cell line cultures[12]. On one hand, we described a new cell culture system that probably could support the infection and replication of HCV viruses. It is well known that HCV investigation has been limited by the lack of an efficient cell culture system permissive for HCV infection and replication. On the other hand, understanding if these small fragments, small EVEs, would be able to produce entire viral particles with infectious proprieties in this cell line is required and this is the major goal of this study. For this purpose, because the small EVEs fragments are endogenous to rabbit and hare genomes, DNA extractions from livers homogenates of one domestic rabbit and one hare, samples used in ours previous work[12], were performed and subjected to RNAse treatment and directly inoculated in naïve MDBK cell cultures. Next, the infectivity of DNA samples and formation of entire HCV-like virus particles in the inoculated naïve MDBK cells were evaluated by immunogold electron microscopy (IEM) with monoclonal antibodies for the NS5 and E2 HCV specific proteins, quantitative Real Time-RT-PCR (qRT-PCR), western blot analysis with monoclonal antibodies for the NS5 HCV specific protein and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF-MS/MS) mass spectrometry. Moreover, phylogenetic analysis of a final consensus constructed sequence for each tested sample revealed the presence of viruses genetically similar to HCV, rabbit and hare HCV-like viruses, tentatively named RHCV and HHCV respectively and that RHCV is closely related to the HCV-1a/HCV-1b genotypes and HHCV is more closely related to the HCV-1b genotype. Our results suggests the possibility that HCV may have been introduced into the human population after their contact with genetic material containing small EVEs homologous to HCV, reinforcing that HCV is not restricted to primates. HCV origin and understanding HCV life cycle are fields that could be better understood with the support on revealed presented data.

## Results

**IEM of rabbit and hare DNA samples.** Cell suspensions of passages (P) P4 and P7 from each MDBK cell flask that were inoculated with DNA from the rabbit and hare liver samples, were evaluated with the use of mouse monoclonal antibodies anti-HCV NS5 protein, and specific immunostaining was demonstrated (Fig. 1). Furthermore, cells suspensions of passage P4 of the tested samples were also evaluated by IEM using mouse monoclonal antibodies anti-HCV E2 protein. Specific immunostaining could be observed including the visualization of HCV-like particles clearly marked by the immunogold particles attached to the HCV E2 protein (Fig. 1). Negative controls (uninoculated MDBK cells) were included and treated with the selected monoclonal antibodies and no reaction was detected (Fig. 1).

**qRT-PCR of rabbit and hare DNA samples.** To evaluate the infectivity and replication of HCV-like particles, HCV RNA titers (in duplicate) were evaluated by qRT-PCR from extracted RNA of the inoculated MDBK cells with rabbit and hare DNA samples at passages P1 and P2. Virus RNA titers were detected at 4.39 log RNA copies $ml^{-1}$ (P1) and 3.75 log RNA copies $ml^{-1}$ (P2) from the rabbit DNA sample and 4.46 log RNA copies $ml^{-1}$ (P1) and 4.61 log RNA copies $ml^{-1}$ (P2) from the hare DNA sample.

**Western blot analysis of HCV NS5B protein.** To further evaluate the infectivity of the HCV-like particles obtained in MDBK cells, new naïve MDBK cells were inoculated with supernatants of the inoculated MDBK cells (with rabbit and hare DNA samples) (P2). A band with a molecular mass of ~65 kDa corresponding to the HCV NS5B protein was detected by the western blot analysis of, both samples (Supplementary Fig. S1). Furthermore, the HCV NS5B protein was undetected in unionoculated MDBK cells (negative control) (Supplementary Fig. S1).

**MALDI-TOF/TOF-MS/MS analysis.** To further confirm the infectivity and efficient HCV-like particles production in MDBK cells, the supernatants of the *de novo* naïve MDBK cells inoculated with rabbit and hare samples (P2) as described above were used. Extracted proteins recovered from the cells were separated by SDS-PAGE and stained with Coomassie Blue protein stain, 7 days post inoculation. The visible protein bands matching HCV specific proteins were selected on the basis of their molecular weight and excised from the gel followed by MALDI-TOF/TOF-MS/MS mass spectrometry analysis as described in methods section. For each digested sample MS/MS spectra were blasted in the MASCOT database search against HCV sequences deposited at UniProt database and a total of 2,338 peptide sequences, such as F, Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B HCV specific proteins, were identified with significant protein scores (P.S.) (P.S. $> 64$ are significant, $p < 0.05$), of which 1,694 and 644 peptide sequences were from the rabbit and hare DNA samples respectively (Table 1, Supplementary Table S2

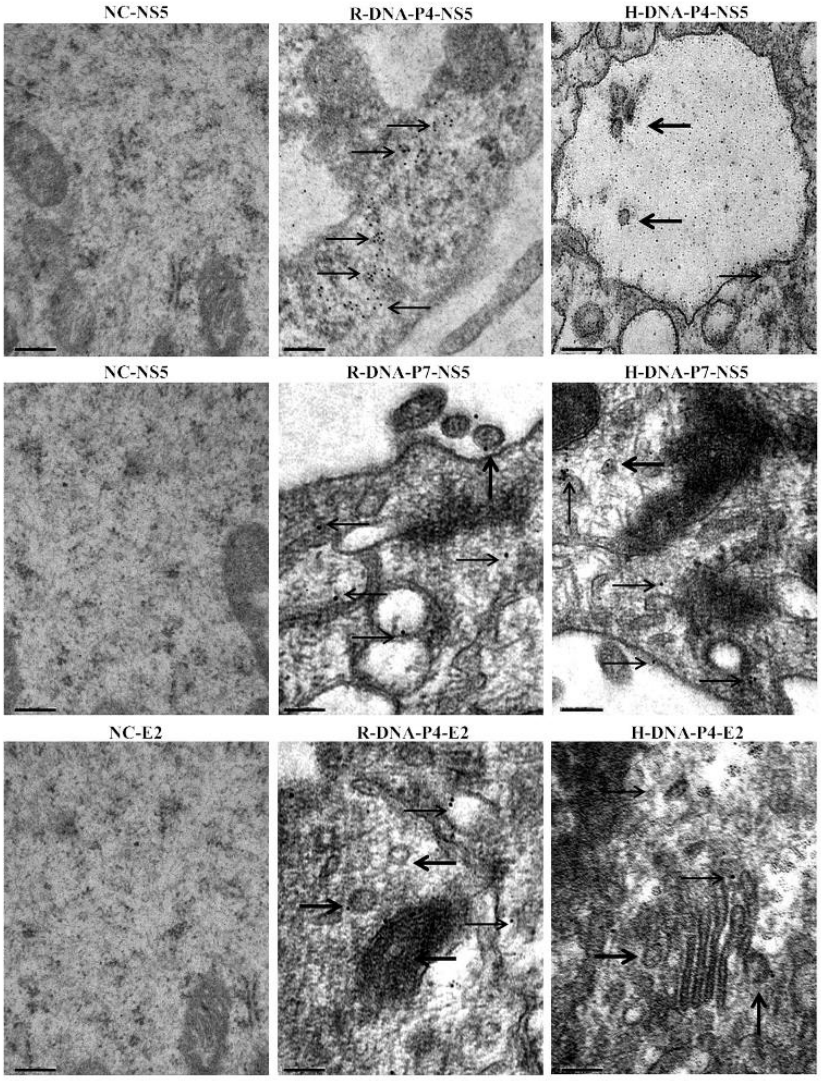


**Figure 1. IEM detection of HCV E2 and NS5 proteins in MDBK cells.** The cells inoculated with DNA extracted from liver homogenates of domestic rabbit and a hare were analyzed 28 (P4, E2 and NS5 proteins) and 49 (P7, NS5 protein) days post infection. These cells and the negative control (NC), uninoculated MDBK cells, were incubated with monoclonal antibodies specific for the HCV E2 and NS5 proteins and specific immunostaining was detected only in inoculated rabbit and hare samples. Visible HCV virus-like particles with specific immunostaining for the HCV E2 and NS5 proteins are indicated by arrows. The immunogold particles were 10 nm in diameter. Scale bars: 200 nm.

and S3). Moreover, a MS/MS peptide sequences summary reported by Mascot version 2.4 for both tested samples presented 51 matches for F, 99 matches for Core, 59 matches for Core/E1, 83 matches for Core/E1/E2, 86 matches for E2, 96 matches for E2/p7/NS2/NS3, 74 matches for NS2, 221 matches for NS3, 99 matches for NS3-4A, 169 matches for NS4B, 338 matches for NS5A, 76 matches for NS5A/NS5B and 253 matches for NS5B HCV specific proteins for rabbit and 44 matches for F, 31 matches for Core, 40 matches for E1, 75 matches for E2, 83 matches for NS3, 79 matches for NS3-4A, 30 matches for NS4A/NS4B, 221 matches for NS5A and 41 matches for NS5B HCV specific proteins for hare samples with significant P.S. can be observed in Table 1. Complete MS/MS peptide sequences reported by Mascot version 2.4 including blast information, such as UniProt accession nos., HCV genotypes, calculated masses (Da), HCV genome positions (amino-acid), P.S., expected-values and peptide sequences for each sample can be

| Animal | UniProt accession no. | HCV Protein (genotype) | Mass (Da) | Protein score | Expect-value | N° of peptide sequence matches |
|---|---|---|---|---|---|---|
| DR | B3TKW7 | F (1a) | 17,015 | 85 | 0.00039 | 51 |
| DR | F4YQ96 | Core | 21,004 | 78 | 0.0019 | 61 |
| DR | B8QB25 | Core | 15,078 | 65 | 0.13 | 38 |
| DR | F1A6I7 | Core/E1 (1b) | 20,996 | 95 | 4.4e-005 | 59 |
| DR | Q98V90 | Core/E1/E2 | 36,052 | 90 | 0.00012 | 83 |
| DR | D1KSI0 | E2 (3a) | 41,959 | 86 | 0.00032 | 86 |
| DR | D3W7L4 | E2/p7/NS2/NS3 (1a) | 54,879 | 82 | 0.00078 | 96 |
| DR | F4YQP9 | NS2 | 24,245 | 69 | 0.015 | 74 |
| DR | F8SI75 | NS3 | 19,174 | 112 | 8e-007 | 60 |
| DR | F6L9J4 | NS3 | 17,754 | 87 | 0.00027 | 57 |
| DR | A3EZJ3 | NS3 | 68,216 | 81 | 0.001 | 94 |
| DR | K7Y470 | NS3-4A | 75,184 | 94 | 4.6e-005 | 99 |
| DR | C9WV93 | NS4B | 27,686 | 97 | 2.5e-005 | 91 |
| DR | Q68586 | NS4B | 20,132 | 82 | 0.00077 | 78 |
| DR | Q6TZ17 | NS5A | 27,433 | 77 | 0.0026 | 78 |
| DR | Q1HFC6 | NS5A | 49,560 | 73 | 0.0061 | 87 |
| DR | A9JKP2 | NS5A | 50,120 | 72 | 0.008 | 85 |
| DR | A9JKN5 | NS5A | 50,064 | 68 | 0.02 | 88 |
| DR | M9UX90 | NS5A/NS5B (3b) | 116,298 | 68 | 0.019 | 76 |
| DR | C7SCB7 | NS5B | 163,51 | 86 | 0.00031 | 72 |
| DR | G8CSB7 | NS5B | 23,529 | 86 | 0.00028 | 75 |
| DR | E7BK75 | NS5B (3b) | 32,573 | 69 | 0.016 | 53 |
| DR | Q81598 | NS5B | 40,858 | 65 | 0.07 | 53 |
| H | B3TL57 | F (1a) | 16,955 | 74 | 0.0053 | 44 |
| H | C0SUM8 | Core (1b) | 46,934 | 77 | 0.0027 | 31 |
| H | B6USQ0 | E1 | 21,278 | 70 | 0.011 | 40 |
| H | E9LLA5 | E2/p7/NS2-3 | 63,556 | 65 | 0.084 | 75 |
| H | D2JVF5 | NS3 | 21,837 | 76 | 0.003 | 51 |
| H | J7HHX1 | NS3 (3) | 15,091 | 70 | 0.012 | 32 |
| H | K7XN61 | NS3-4A | 75,514 | 83 | 0.0007 | 79 |
| H | Q81592 | NS4A/NS4B | 13,899 | 65 | 0.14 | 30 |
| H | Q1HFF4 | NS5A | 48,917 | 68 | 0.019 | 56 |
| H | Q1HFF3 | NS5A | 48,940 | 67 | 0.025 | 52 |
| H | A4UXV5 | NS5A (1b) | 49,355 | 65 | 0.052 | 56 |
| H | Q1HFG0 | NS5A | 49,009 | 65 | 0.047 | 57 |
| H | I0J2K8 | NS5B | 12,668 | 76 | 0.0032 | 41 |

**Table 1. MALDI-TOF/TOF-MS/MS mass spectrometry.** MS/MS peptide sequences summary reported by Mascot version 2.4 of *de novo* inoculated new naïve MDBK cells using supernatant of first inoculated naïve MDBK cells with rabbit and hare DNA samples, 7 days post inoculation. Protein score > 64 are significant ($p < 0.05$). DR - Domestic rabbit (Oryctolagus cuniculus), H - Hare (Lepus europaeus).

observed in Supplementary Table S1 and S2 for tested rabbit and hare DNA samples inoculated in MDBK cells respectively. Furthermore, different HCV genotypes matched animal samples genetic sequences when they were blasted against HCV. Identified peptide sequences that matched with unidentified, 1a, 1b, 3a, 3b HCV genotypes and unidentified, 1a, 1b and 3 genotypes can be observed for rabbit and hare samples respectively (Supplementary Table S2 and S3). The spectra generated by MS/MS analysis of all identified peptide sequences for both tested animal samples can be observed in Supplementary Fig. S2. Proteins extracted from uninoculated MDBK cells (negative control) were also separated by SDS-PAGE procedures and stained with imperial protein stain and no bands with similar molecular mass to HCV proteins were observed.

When blastp of constructed rabbit HCV-like viruses amino acids consensus sequences for specific HCV proteins were conducted in the UniProt database (http://www.uniprot.org/blast/), similarities of 100%–81%; e-values of 0.0–$1.0 \times 10^{-139}$, including different HCV genotypes/subtypes, were shared for core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B HCV specific proteins (Supplementary Table S5). The final RHCV amino-acid consensus sequence shared the genome polyprotein of HCV-1a and -1b genotypes with similarities of 78%–77% and e-values of 0.0–0.0 (Supplementary Table S5). Moreover, the constructed hare HCV-like viruses amino-acid consensus sequences for specific HCV proteins, shared similarities of 100%–78%; e-values of 0.0–$8.0 \times 10^{-166}$, including different HCV genotypes/subtypes, for core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B HCV specific proteins. The final HHCV amino-acid consensus sequence, shared with genome polyprotein of HCV, HCV- 1a and -1b genotypes similarities of 72%–70% and e-values of 0.0–0.0 (Supplementary Table S6). This data supported the selection of the HCV genotypes/subtypes used in the phylogenetic analysis.

**Phylogenetic analysis of RHCV and HHCV.** Phylogenetic relationships were inferred using the NJ and the ML methods as described in methods section. RHCV and HHCV were phylogenetically classified by determining the genetic relatedness to representative viruses of genus *Hepacivirus*. Notably, this phylogenetic analysis, using both methods, placed RHCV and HHCV as more closely related to the seven HCV genotypes, supported by strong bootstrap values of 99%–100% (of 1,000 replicates) (Fig. 2). CHV, NPHV, and RHV; GHV and BHVcladeA formed a second and third cluster respectively, both also supported by a strong (100%) bootstrap value of 1,000 replicates (Fig. 2). Furthermore, the identified RHCV is more closely related to HCV-1a genotype and the identified HHCV is more closely related to HCV-1b genotypes (Fig. 2). The phylogenetic analysis clearly demonstrates that these newly produced rabbit and hare HCV-like are more closely related to HCV than the described animal hepacivirus used in this analysis. In this study, the phylogenetic analysis of known hepaciviruses CHV, NPHV, RHV, GHV and BHVcladeA, showed clustering concordance with data previously described[13–15]. These results clearly suggest that entire HCV-like particles are produced after the inoculation of rabbit and hare DNA in MDBK cells, which is in accordance with the visualization by IEM of entire HCV-like particles.

## Discussion

In our previous study, the presence of endogenous HCV homolog fragments in wild/domestic rabbits (*Oryctolagus cuniculus*) and hare (*Lepus europaeus*) genomes and their capacity to replicate in MDBK cell cultures were detected by PCR and RT-PCR and MALDI-TOF/TOF-MS/MS analysis[12]. We have demonstrated that several homolog fragments to different HCV specific proteins are integrated in the rabbit and hare genomes[12]; (see Table 1 and 2 from this reference). Furthermore, we showed by blastn between HCV-1b (ID - D90208, HCV database; individual proteins and polyprotein) and *O. cuniculus* and by blastn between HCV specific primers (used for PCRs procedures) and *O. cuniculus*, that the sequences matched with high degree of homology (100%–91%) with several distinct scaffold or chromosomes in the rabbit genome. Some of the homolog base pair sequences found within the same fragment matched sequences that appeared repeatedly in several distinct scaffold or chromosomes[12], (see Table S4 and S6 from this reference). Moreover, the results obtained have showed that all these small endogenous fragments homologous to HCV-like proteins are integrated in the rabbit and hare genomes and were detected in high copy numbers in the studied samples[12]. These findings lead us to hypothesise that the rabbit and the hare have small EVEs homologous to all HCV genetic specific regions that, when exposed to a suitable biological substrate, in these case the MDBK cell line, are able to internalize the cells and initiate its own replication using the host cell machinery to produce proteins and polyproteins and likely entire HCV-like particles.

EVEs derived from a flavivirus-related RNA involving a gene sequence of the virus RNA that replicates using an RNA-dependent RNA polymerase integrated into the Aedes spp. mosquitoes genome was previously described[7]. Based in these findings and in the functional pathways of ssRNA+ viruses genomes, such as, the virion (genomic) RNA is the same sense as mRNA and therefore can function as mRNA that can be immediately translated by host cell machinery upon infection, working the genomic RNA as template for viral RNA replication[1–3], we decided to look at the functional and biological significance of these genetic elements, smalls EVEs HCV homolog's, present in the rabbits and hares genomes.

The evidence that HCV cell entry requires essential receptors or coreceptors like human cluster of differentiation 81, scavenger receptor class B type I, claudin 1 and occluding were previously described[16–20]. The expression of claudin 1 in bovine kidneys, a sequence of the bovine scavenger receptor class B type I with close identity to human sequences, occludin, that have been identified in high concentrations in tight junctions of the MDBK cell line, and that cluster of differentiation 81 is expressed in several bovine tissues including the liver and Kidney were also previously reported[21–25] reinforcing our present research.

In this study, we showed that RHCV and HHCV are produced upon the inoculation of MDBK cells with rabbit/hare genomic DNA, and that the resulting RHCV is closely related to HCV-1a/HCV-1b genotypes and HHCV is more closely related to HCV-1b genotype. Our conclusions are supported by the following observations. First, we demonstrated that entire rabbit and hare HCV-like particles are formed in MDBK cells upon the inoculation of naïve MDBK cells with DNA extracted from domestic rabbit and from a hare. HCV-like particles were demonstrated by IEM and with the use of specific immunostaining that detected the NS5 and E2 HCV proteins. Immunogold particles were detected in

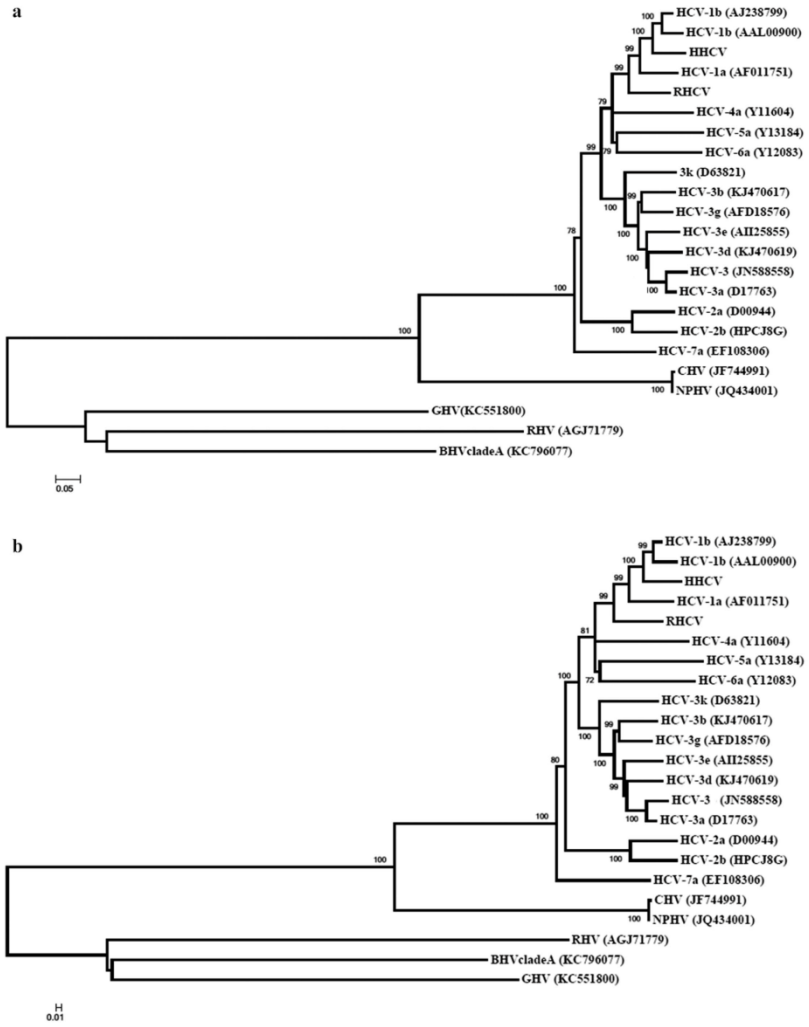


**Figure 2. Phylogenetic analysis of RHCV and HHCV.** Phylogenetic analysis using partial CDS sequences of reported HCV-like viruses recovered from *de novo* naïve MDBK cells inoculated with supernatants of the first rabbit and hare DNA samples inoculated in naive MDBK cells. (**a**) NJ and (**b**) ML methods were inferred. Bootstrap resampling was used to determine the robustness of branches; values of $\geq 72\%$ (from 1,000 replicates) are shown. Reported HCV-like viruses are RHCV (rabbit) and HHCV (hare) respectively. A listing of host species, virus abbreviations and original accession numbers for sequences used in the phylogenetic trees are provided in Supplementary Table S3.

several cellular organelles of infected cells such as vacuoles, lipid droplets, mitochondria and Golgi apparatus in agreement with previous observations[12,26,27]. Secondly, we detected HCV RNA titers of 4.39 log copies $ml^{-1}$ (P1), 3.75 log copies $ml^{-1}$ (P2) for the rabbit DNA passages in cells; 4.46 log copies $ml^{-1}$ (P1), 4.61 log copies $ml^{-1}$ (P2) for the hare DNA cell passages when targeting the HCV 5′ untranslated region by qRT-PCR. Thus, the obtained HCV RNA titers indicates the production of entire HCV-like particles in the inoculated cells which is in accordance to data previously described, when the production of infectious HCV-1a genotype was evaluated in cultured human hepatoma cells[28]. Thirdly, we were able to detect NS5B HCV specific protein in *de novo* inoculated naïve MDBK cells by western blot analysis. These results demonstrate that the generated HCV-like viruses have the capacity to infect the *de novo* inoculated naïve MDBK cells, using both types of samples. Finally, the identification of several peptide sequences that matched with all specific structural and nonstructural HCV proteins by

MALDI-TOF/TOF-MS/MS analysis of each tested sample, in the *de novo* inoculated naïve MDBK cells, allowed its genetic relationship with HCV. The final consensus of the amino-acid sequence construction resulted in rabbit RHCV and hare HHCV amino-acid sequences, consisting of almost all HCV polyproteins. Herewith, RHCV and HHCV sequences were identified and classified as genetically related to HCV, supported by the obtained blastp results of the identified individual and final consensus sequences of HCV proteins in the UniProt database and by phylogenetic analysis. Moreover, RHCV was more closely related to HCV-1a/HCV-1b genotypes and HHCV was more closely related to HCV-1b genotype. Furthermore, the newly formed and identified HCV-like viruses are the most closely related to HCV genotypes described to date, when compared with others HCV-like viruses detected in the dog, horse, rodents or bats[13–15].

For the best of our knowledge, this is the first report on the generation of HCV-like viruses in a cell culture system. We clearly demonstrate that entire HCV-like particles are produced in MDBK cell line, suggesting that the small EVEs present in the genomic DNA of the rabbit and hare can internalize the MDBK cells and together with the cell machinery initiate replication and the generation of novel HCV-like virus, and that probably HCV could also be generated likewise. However, the detailed mechanisms of this process and a comprehensive knowledge of the biological and medical significance of our findings should be further investigated. This could be a breakthrough in clarifying the theories of the HCV origin, evolution, the improvement of the HCV life cycle understanding and the opening of new horizons to predict/evaluate new therapeutic approaches for this dangerous pathogen.

## Methods

**Samples.** Total DNA extracted from liver homogenates of one domestic rabbit *(Oryctolagus cuniculus)* and one hare *(Lepus europaeus),* samples used in our previous study[12]. All legal requirements and permits by the Instituto da Conservação da Natureza e da Biodiversidade (ICNB) (the national authority for nature conservation and wildlife protection) for rabbit and hare specimens collection and use were followed. The ICNB permit is issued under the EU Habitats Directive, considering safety measures to avoid spread of any pathology, namely the disinfection of equipment with 1% hypochlorite solution of dead animal species. All experimental protocol were carried out in accordance with the approved guidelines by the Portuguese Veterinary Authority of the Ministry for Agriculture, Sea, Environment and Spatial Planning (Decree law n.° 113/2013 of 7$^{th}$ August 2013) were the current European Communities Council Directive of September 2010 (2010/63/UE) is present. The methods were carried out in accordance with the approved guidelines.

**Cell cultures.** MDBK cell line (American Type Culture Collection) obtained from the Friedrich-Loeffler-Institute (Insel Riems, Germany) were maintained as previously described[12].

**DNA extraction from liver homogenates samples.** Total DNA was extracted from 10% (w/v) liver homogenates samples treated with RNAse A using the QIAamp® DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. DNA, RNA and protein concentrations were measured before sample inoculation in MDBK cells in a Qubit® Fluorometer (Invitrogen, Carlsbad, USA) according to the manufacturer instructions (Supplementary Table S1).

**Rabbit and hare liver DNA inoculation in naïve MDBK cells.** Samples inoculations were processed according to procedures previously described[12]. Briefly, cell culture medium of a T25 cell culture flask with MDBK cells (80% in confluence) was discarded and then inoculated with extracted DNA (200 µl; 2.5 ng ml$^{-1}$) of rabbit liver homogenate and incubated at 37 °C and 5% $CO_2$ for 4 h (adsorption). After, the cell monolayer was washed twice with 1X phosphate buffered saline (PBS), replaced with new cell culture medium and maintained at 37 °C and 5% $CO_2$ for 7 days. Then, 7 passages were performed subsequently, with 7days incubation period between each passage. For the hare DNA (200 µl; 640 ng ml$^{-1}$) sample the same procedure as for rabbit was undertaken. Cells were evaluated for HCV detection at indicated passages post inoculation by IEM and qRT-PCR analysis. Uninoculated MDBK cells were maintained and included as negative controls in all procedures.

**MDBK cells *de novo* inoculated with supernatants recovered from the first MDBK cells inoculation with DNA samples (liver).** To further evaluate the infectivity and efficient production of HCV-like particles, supernatants of previously inoculated MDBK cells with liver DNA samples were used as inoculums in *de novo* naïve MDBK cells. For this, 2 ml of supernatant filtered through a 0.2 µm membrane obtained from passage P2 was adsorbed onto T150 tissue culture flasks with fresh naïve MDBK cells, 80% in confluence, and incubated at 37 °C and 5% $CO_2$ for 4 h. After, the monolayers were washed twice with 1X PBS, the culture medium was replaced and flasks were maintained at 37 °C and 5% $CO_2$ for 7 days. The supernatants of each flask were then evaluated at 7 days post inoculation by MALDI TOF/TOF-MS/MS analysis. Uninoculated MDBK cells (negative controls) were maintained and equally performed as samples.

**Immuno electron microscopy.** For IEM, the cell suspensions of passages P4 and P7 of each MDBK cell flask inoculated with liver DNA of rabbit and hare together with the negative controls (uninoculated cells) were processed according to procedures previously described[12] with one modification. After the blocking process with BSA (Sigma-Aldrich, Steinheim, Germany) in Tris Buffered Saline, the grids were incubated for 60 min on a drop of specific monoclonal antibodies for the NS5 (P4 and P7) and E2 (P4) HCV specific proteins.

**RNA extraction and HCV RNA quantification by qRT-PCR.** Cell lysates from passages P1 and P2 were of the inoculated cells with rabbit and hare DNA, were collected and subjected to three freeze/thaw cycles at −80 °C / room temperature. Total RNA extractions were performed using the QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) following the manufacturer instructions with some modifications. Briefly, 100 μL of treated supernatants and cells plus 5 μL of internal control (provided in HCV Real-TM Quant kit) were extracted and RNAs were eluted with 50 μL of buffer AVE. After, the HCV RNA titers (in duplicate) from extracted RNA were determined by qRT-PCR targeting the HCV 5′ untranslated region, HCV Real-TM Quant kit (Sacace Biotechnologies Srl, Como, Italy), according to the manufacturer's instructions in a StepOne™ Real-Time PCR System (Applied Biosystems, Foster, California). Uninoculated MDBK cells maintained as negative controls were treated as samples.

**Western blot of HCV NSB Protein.** Western blot procedures were performed as previously described[12,29] with some modifications. In brief, total protein extracts from supernatants at 7 days post inoculation recovered from new naïve MDBK cells inoculated with rabbit and hare samples and an uninoculated naïve MDBK cells (negative control) were mixed with sodium dodecyl sulfate reducing buffer, denatured for 5 minutes (boiling), and loaded onto a 12% precast sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad, Hercules, USA). After electrophoresis, proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, USA) for 1 hour at 100 V. The membrane was blocked for 1 hour with 5% nonfat dry milk (Molico-Nestlé, Vevey, Switzerland) in tris buffered saline with 0.05% Tween 20 (Merck, Darmstadt, Germany) at room temperature. The membrane was incubated overnight with mouse monoclonal antibodies anti-HCV NS5 (Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (1:100) and subsequently incubated with goat anti-mouse IgG-alkaline phosphatase (Sigma-Aldrich, Saint Louis, USA) (1:200) antibody for 60 minutes. Finally, proteins were visualized by chemiluminescence using an ECF substrate (GE Healthcare Amersham, Freiburg, Germany).

**MALDI-TOF/TOF-MS/MS mass spectrometry in *de novo* inoculated MDBK cells.** The supernatants (7 days) recovered from the new naïve MDBK cells inoculated with rabbit and hare samples, as described above, were harvested and filtered through a 0.2 μm membrane, for protein extraction and MALDI TOF/TOF-MS/MS analysis as previously described[12,30] with one modification. The mass spectrometry approaches were performed in a 4800 Plus MALDI TOF/TOF Analyzer (AB SCIEX, Framingham, MA). MS data was further treated to obtain a consensus sequence from the rabbit and hare DNA samples as described below. Uninoculated MDBK cells (negative control) were also included for protein extraction and separated by SDS-PAGE and stained with imperial protein stain. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (http://www.ebi.ac.uk/pride/archive/) partner repository with the dataset identifier PXD001992.

**Drawing Consensus Sequences.** Two final consensus sequences of the almost complete HCV-like viruses polyprotein, rabbit (RHCV) and hare (HHCV) HCV-like viruses, were constructed using recovered peptide sequences from *de novo* inoculated naïve MDBK cells determined by MALDI TOF/TOF-MS/MS analysis. Thus, these final consensus sequences for RHCV and HHCV were obtained by complete sequences and by consensus sequences construction of the significant peptide sequences identified for the different HCV-like structural and non-structural proteins that matched with several UniProt accession nos. Therefore, core protein (F protein included) consensus sequence was constructed using recovered peptide sequences that matched with B3TKW7, F4YQ96 B8QB25 and F1A6I7, E1 protein using peptide sequences that matched with Q98V90, E2 protein using peptide sequences that matched with D1KSI0, p7 protein using peptide sequences that matched with D3W7L4, NS2 protein using peptide sequences that matched with F4YQP9, NS3 protein using peptide sequences that matched with F8SI75, F6L9J4, A3EZJ3 and K7Y470, NS4A protein using peptide sequences that matched with K7Y470, NS4B protein using peptide sequences that matched with C9WV93 and Q68586, NS5A protein using peptide sequences that matched with Q6TZ17, Q1HFC6, A9JKP2, A9JKN5 and M9UX90 and NS5B protein using peptide sequences that matched with M9UX90, C7SCB7, G8CSB7, E7BK75 and Q81598 to obtained the RHCV final consensus sequence. Moreover, to obtain the HHCV final consensus sequence, core protein (F protein included) consensus sequence was constructed using recovered peptide sequences that matched with B3TL57 and C0SUM8, E1 protein using peptide sequences that matched with B6USQ0, E2 protein using peptide sequences that matched with E9LLA5, p7 protein using peptide sequences that matched with E9LLA5, NS2 protein using peptide sequences that matched with E9LLA5, NS3 protein using peptide sequences that matched with D2JVF5, J7HHX1 and K7XN61, NS4A protein using peptide sequences that matched with Q81592, NS4B protein using peptide sequences that matched with Q81592, NS5A protein using peptide sequences that matched with Q1HFF4, Q1HFF3, A4UXV5 and Q1HFG0

and NS5B protein using peptide sequences that matched with I0J2K8. For more detailed information consult Table 1, Supplementary Table S2 and S3. The consensus sequences constructed for each specific HCV-like particle proteins relatively for each animal and the final constructed consensus sequence of HCV-like viruses obtained for each animal were used to perform blastp in the UniProt database (http://www.uniprot.org/blast/). The final constructed amino-acid sequences of RHCV and HHCV have been deposited in the ProteomeXchange Consortium via the PRIDE (http://www.ebi.ac.uk/pride/archive/) partner repository with the dataset identifier PXD001992.

**Phylogenetic analysis.** The amino acids sequences of draw almost complete RHCV and HHCV polyproteins obtained from complete sequences and consensus sequences construction of all peptide sequences recovered from *de novo* inoculated naïve MDBK cells by MALDI TOF/TOF-MS/MS procedures with significant protein scores (P.S.) to HCV specific proteins, were aligned using Clustal W through MEGA version 5.05[31,32] for phylogenetic inference. An evolutionary history was inferred using the Neighbor-Joining (NJ) method[33]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches[34]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method[35] and are in the units of the number of amino-acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). The analysis involved 23 amino-acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 1,554 positions in the final dataset and the evolutionary analyses were conducted in MEGA version 5.05[32]. An evolutionary history using the Maximum Likelihood (ML) method was also inferred. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches[34]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Whelan and Goldman method[36] and are in the units of the number of amino-acid substitutions per site. The analysis involved 23 amino-acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 1,554 positions in the final dataset and evolutionary analyses were conducted in MEGA version 5.05[32]. For a precise classification of the first rabbit and first hare HCV-like viruses, the complete coding sequences (CDS) of 16 HCV genotypes/subtypes sequences deposited at GenBank, NCBI (http://www.ncbi.nlm.nih.gov/) database, as summarized in Supplementary Table S4, were included in the phylogenetic analysis. In addition, CDS sequences of hepaciviruses (HCV-like viruses) achieved in animal species (dog, horse, *Peromyscus maniculatus* (rodent), *Hipposideros vittatus* (bat) and black-and-white *Colobus* were included (Supplementary Table S4).

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## 3.4 - The Replication of Synthetic Fragments Homolog to HCV and to the *O. cuniculus* Genome in MDBK Cell Culture: Preliminary Results

# The Replication of Synthetic Fragments Homolog to HCV and to the *O. cuniculus* Genome in MDBK Cell Culture: Preliminary Results

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## Abstract

Our previous findings demonstrating that endogenous HCV homolog fragments present in the European rabbit (*O. cuniculus*) and European hare (L. europaeus) genomes can replicate in Madin-Darby bovine kidney (MDBK) epithelial cell, as well as that Hepatitis C-like viruses are produced in the same cell line upon its inoculation with European rabbit and European hare DNA, led us to investigate if synthetic fragments with total homology between core, E2 and NS4B HCV proteins and the European rabbit genome have the ability to internalize and replicate in naïve MDBK cell line. The efficient internalization and replication of the selected tested samples could be demonstrated by virus titers detection using the quantitative real-time RT-PCR analysis.

## Text

Unmodified or modified HCV genomic RNA belonging to different HCV genotypes have been synthesized, cloned and then transfected into different cell lines, such as hepatoma cell line (Hep) or Hep3B cells, resulting in the production of infectious viral particles or pseudoparticles (1, 2, 3). It was reported that unmodified genomic RNA derived from the HCV-2a JFH-1 strain produced infectious virions after transfection into Huh-7 hepatoma cells (1) and that modified synthetic RNA derived from a prototype HCV-1a H77 strain containing five adaptive mutations also shows an efficient replication in Huh-7 cells after transfection (2, 3). Moreover, a study on computational reconstruction of a representative synthetic HCV-1a (bole1a) pseudoparticle that robustly infect Hep3B cells was described (4), as it was also

demonstrated that the fully synthetic HCV genome contains representative epitopes and envelope genes that assemble properly and mediate entry into target cells.

Following our previous description on the capacity that endogenous HCV homolog fragments present in the European rabbit and European hare genomes, can naturally replicate in MDBK cell line (5) and that Hepatitis C-like viruses are produced in MDBK cell line upon its natural inoculation with European rabbit and European hare DNA (6), we investigated the capacity of some samples of synthesized fragments showing total homology between core, E2 and NS4B HCV proteins and the *O. cuniculus* genome, to passively internalize and replicate in the naïve MDBK cell line. Samples of synthetic fragments with total homology between core, E2 and NS4B HCV proteins and the *O. cuniculus* genome (Table 1) were inoculated into MDBK cell line according to procedures previously described (5). The cell culture medium of T25 cell culture flasks with MDBK cells (80% in confluence) was discarded and cells were inoculated with the synthesized samples (one sample per flask) and incubated at 37ºC and 5% $CO_2$ for 4h. After this incubation period cell monolayer's were washed twice with 1X phosphate buffered saline (PBS), and the new cell culture medium replaced and flasks were maintained at 37ºC and 5% $CO_2$ for 7 days. Subsequentially, four cell passages were performed. Uninoculated MDBK cells were maintained and included as negative controls in all procedures. The supernatants from passage 4 (P4) were used for total RNA extractions using the QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and HCV RNA titers determined (in duplicate) by quantitative real-time RT-PCR analysis (qRT-PCR), HCV Real-TM Quant kit (Sacace Biotechnologies Srl, Como, Italy), according to the manufacturer instructions in a StepOne™ Real-Time PCR System (Applied Biosystems, Foster, California). Virus RNA titers of ~3-5 log RNA copies $ml^{-1}$ were detected in the tested samples (Table 2) demonstrating that the inoculated samples of synthetic fragments could passively infect and replicate in MDBK cells. Uninoculated MDBK cells (negative controls) were also tested by qRT-PCR and no positive signal was detected. However, further studies that include these and other synthetic fragments as well as other methodologies are to be performed to further confirm these findings. Furthermore, the related host-MDBK cell line factors should also be investigated. Our preliminary results indicate that research in the

fields of HCV pathogenesis, new diagnostic tools, new antiviral targets or vaccine production could be further improved applying these strategies.

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## Tables

**Table 1.** Synthetic fragments with total homology between core, E2 and NS4B HCV proteins and the *O. cuniculus* genome.

| HCV protein | Synthetic fragments |
|---|---|
| Core 186 | 5` - ATGTACCCCATGAGGTCGGC - 3` |
| Core 134 | 5` - CCAAGAGGGACGGGAACCTC - 3` |
| E2 | 5` - GCTGTCATTACAGTTAAGGGCA - 3` |
| NS4B | 5` - CCCACTGACAAAGTTCCACAT - 3` |

**Table 2.** HCV RNA titers evaluated by qRT-PCR of inoculated MDBK cells (P4) with synthetic fragments with total homology between HCV and the *O. cuniculus* genome**.**

| Synthetic fragments homologous to HCV | HCV RNA titer (log copies $ml^{-1}$) |
|---|---|
| Core protein 186 | ~ 5 |
| Core protein134 | ~ 3 |
| Envelope | ~ 3 |
| NS4B | ~ 3 |

# CHAPTER 4

# BOVINE CELL MODEL FOR WILD TYPE HCV REPLICATION

# 4.1 - Naïve Cell Models for HCV Infection and Replication: Preliminary Results

# Naïve Cell Models for HCV Infection and Replication: Preliminary Results

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## Abstract

Hepatitis C virus is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. We recently described that endogenous HCV homolog fragments can replicate in Madin-Darby bovine kidney epithelial cell line and bovine testis primary cells. Here, we show that both bovine cell cultures can also sustain HCV infection and replication after inoculation with patients infected sera, HCV uncharacterized (HCVuc), -1a and -2c genotypes using molecular biology, immunofluorescence and immunogold electron microscopy methods.

## Text

Hepatitis C virus (HCV) is positive-sense RNA enveloped virus of the *Flaviviridae* family and it is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide (1). A detailed understanding of the virus life cycle, the development of new antivirals or a vaccine is urgent. HCV infects and replicates in liver hepatocytes of infected patients, and there was a breakthrough when Wakita *et al.* (2) described a HCV clone capable of replicating in Huh 7 cells. However, these cell culture systems among others like Vero cells and mosquito cells were not reliable when used in antiviral or molecular HCV studies (3-6). We recently described that endogenous HCV homolog fragments can efficiently replicate in naïve

bovine cell cultures, Madin-Darby bovine kidney (MDBK) epithelial cell line and bovine testis (BT) primary cells (7), respectively, and this could be a step forward in HCV research or antivirals and vaccine development.

HCV cell entry requires essential receptors or coreceptors like human cluster of differentiation 81 (CD81) (8), scavenger receptor class B type I (SR-BI) (9), claudin 1 (CLDN1) (10) and occludin (OCLN) (11). In a recent study it was described that CD81 is expressed in several bovine tissues including kidney and liver (12). Expression of CLDN1 in bovine kidneys suggesting distinct characteristics in the tight junctions (TJ) (13) as well as a sequence of the bovine SR-BI HDL-receptor with a close identity to human sequences (14) have been also reported. OCLN, identified and detected in high concentration in TJ of MDBK and chick intestinal epithelial cells (15, 16) was also described. OCLN is an essential indicative for HCV infection of naturally permissive cells (11) and recently it was reported that the human OCLN is a HCV entry factor required for infection of mouse cells (11). Moreover, low density fractions from serum of patients infected with HCV established the physical association within lipo-viro-particle of apolipoproteins (B, CI, CII, CIII), HCV RNA and envelope glycoprotein's E1 and E2 (17). Also, NS3 to NS5B proteins establish membrane bound replication complexes that catalyze RNA replication and NS5B protein codes RNA-dependent RNA polymerase which serves to replicate the HCV-RNA genome (18-20).

To evaluate HCV infectivity, MDBK and BT cell cultures inoculated with HCV-1a, -2c and HCVuc infectivity titers were determined. The HCV-1a and -2c genotypes serum samples were obtained from HCV-infected patients, irreversibly anonymized at the Santo António General Hospital, Porto, Portugal; the used protocol was approved by the Ethics Committee of the Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), University of Porto, Porto, Portugal (CETI; Projeto Nº 086/2014); and HCVuc is a HCV positive control, included in a kit INNO-LIA$^{TM}$ HCV score (Innogenetics, Ghent, Belgium). MDBK and BT cells were maintained as previously described (7). RNA extractions from harvested cell cultures (supernatants and cells) from all inoculated samples, at the defined time points, were subjected to three freeze/thaw cycles at -80 º C/room temperatures were performed using the QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) following the manufacturer instructions with some modifications. Briefly, RNA from cell lysates (100 µl) with 5 µl of internal control

(provided in HCV Real-TM Quant kit) were extracted and RNAs were eluted with 50 µl of buffer AVE. HCV RNA titers from extracted RNA were measured at the defined time points by a 5` UTR quantitative Real Time-PCR (qPCR), HCV Real-TM Quant kit (Sacace Biotechnologies Srl, Como, Italy) according to the manufacturer instructions in a StepOne™ Real-Time PCR System (Applied Biosystems, Foster, California).

For BT and MDBK cell cultures infected with HCVuc stock (1.24E+03 IU/ml) for 336h and 1008h, respectively, HCV RNA titers (mean of two determinations) ranged from $10^3$ to $10^5$ and from $10^3$ to $10^6$ IU/ml, respectively. Moreover, HCV RNA titers (mean of two determinations) ranged from $10^3$ to $10^4$ IU/ml and $10^4$ IU/ml were determined in MDBK cells inoculated with genotypes -1a (3.76E+03 IU/ml) and -2c (1.18E+04 IU/ml) stocks for 336h, respectively. To further characterize the different virus's infectivity patterns, kinetic experiments in MDBK and BT cell cultures were performed. After inoculation of MDBK and BT cell cultures with HCVuc stock, a ~3 log increase in HCV RNA titers to a peak titer of $10^6$ IU/ml was observed (Fig. 1A, 1B). However, when MDBK cell cultures were inoculated with HCV-1a stock and HCV-2c stock a ~1 log increase in HCV RNA titers raised to peak titers of $10^4$ and $10^5$ IU/ml, respectively, was observed (Fig. 1C). Interesting aspects of the use of MDBK and BT culture systems are that even starting with low inoculums concentration, the virus concentration for the studied samples was continuously high with little variation for incubation period and a virus livelihood is also observed. Lázaro *et al*. (21) were able to demonstrated that human fetal hepatocyte cultures can be infected by patient serum of various HCV genotypes, HCV-1a included, but only about 80% of the tested samples were able to infect these cell cultures. A study using MDBK and others cell cultures infected with HCV-3a were recently described (22) but the authors were not able to demonstrated HCV-3a infection in MDBK cells. However, ours results demonstrate the infectivity of different HCV genotypes in MDBK cells and we previously demonstrated that homolog HCV fragments with high homologies to different HCV genotypes, HCV-3a included, also replicate in these cells.

To investigate the infectivity and replication of inoculated HCV-positive samples in MDBK and BT cell cultures IFA and IEM assays were performed using mouse monoclonal antibodies (MAbs) anti-HCV NS3, anti-HCV NS4A and anti-HCV

NS5 as previously described (7). MDBK cell cultures were infected with HCVuc stock for 336h and 1008h, respectively. For both inoculated cell cultures, cells were harvested at 336h post infection (p.i) and tested by IFA and IEM using mouse MAbs anti-HCV NS3, anti-HCV NS4A and anti-HCV NS5 and specific immunostaining for the three selected antibodies could be demonstrated (Fig. 2A, 2B). Negative controls (uninoculated MDBK and BT cells) that have been treated with the selected MAbs were included and no reaction was detected (Fig. 2A, 2B). Immunogold particles were detected in different cell organelles such as nucleous, mitochondria and cytoplasm (Fig. 2B) consistent with others reports previously described for HCV replication in cell cultures (23, 24). Immunogold particles were also detected in TJ when MAbs anti-HCV NS5 (Fig. 2B) were used suggesting high relation with the HCV cell entry and OCLN that is highly concentrated in these intercellular junctions. MDBK cells were also infected with HCV-1a and HCV-2c stocks for 336h. Harvested cells using MAbs anti-HCV NS3 (16 and 48h) and anti-HCV NS5 (16, 48 and 96h) were tested by IFA and specific immunostaining for the two selected MAbs could be observed (Fig. 3A, 3B). Negative controls (uninoculated MDBK cells) treated with the selected MAbs were included and no reaction was detected (Fig. 3A, 3B).

The presence of the essential HCV cell entry factors in bovine tissues (e.g. kidney, liver, MDBK cells), as described above, and the obtained results by IFA, IEM and qPCR for the studied samples successfully demonstrate the permissibility of the used bovine cell cultures to HCV. Among this, we suggest the bovine cell cultures, in particular MDBK cell line, as a new efficient *in vitro* model for studies of HCV pathogenesis, for the development of effective antivirals and vaccines as tools for therapeutics and prevention of this disease.

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## Figures

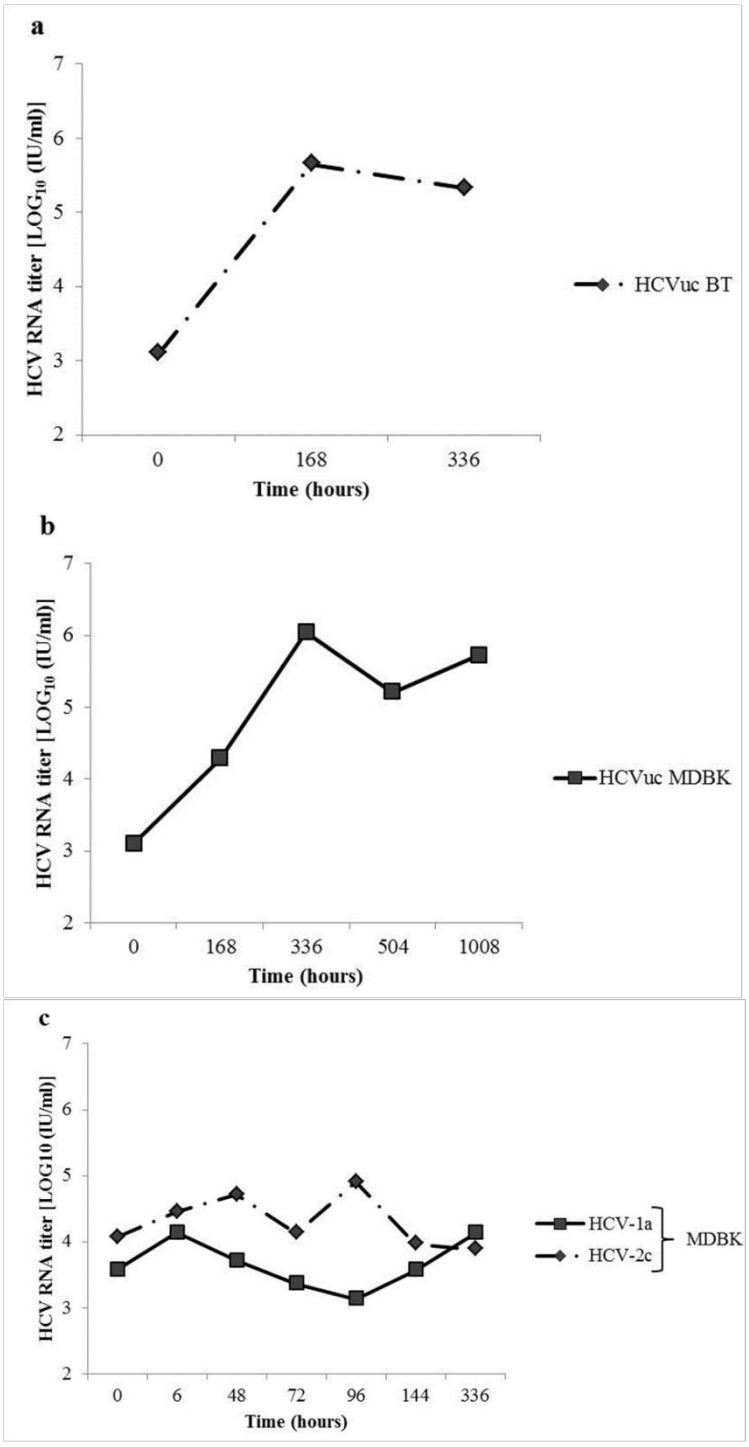


**Figure 1.** Comparative kinetics studies of intergenotypic viruses of HCVuc, HCV-1a and HCV-2c. MDBK and BT cells were infected with the respective stock virus for several weeks and HCV RNA titers were detected in harvested supernatants and cells by qPCR. a, HCVuc, BT infected cells at 168 and 336h post infection. b, HCVuc, MDBK infected cells at 168 and 336h p. i. c, HCV-1a and -2c, MDBK

infected cells at 6, 48,72, 96, 144 and 336h p. i. HCV RNA titers were measured under the detection limit of the used test.

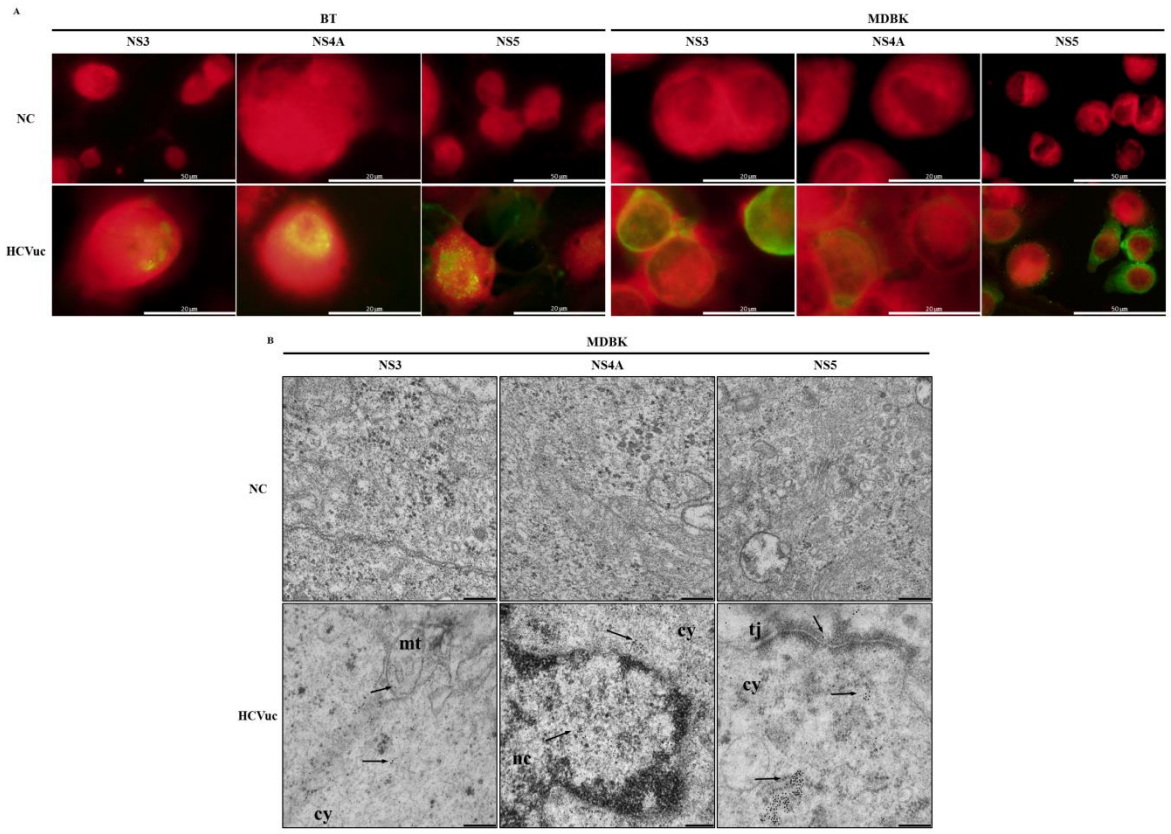


**Figure 2.** Detection of HCV proteins by IFA and IEM in BT and MDBK cell cultures infected with HCVuc. BT and MDBK cell cultures infected with serum from HCV-infected patient, HCVuc, harvested at 336h p. i. a, Both cell cultures tested by IFA using mouse MAbs anti-HCV NS3, anti-HCV NS4A and anti-HCV NS5. Scale bars: 50 μm (BT – NS3 and NS5 NC, MDBK – NS5 NC and HCVuc) and 20 μm (others). b, MDBK cell cultures tested by IEM using the same MAbs as in IFA**.** The immunogold particles were detected in mitochondria (mt), cytoplasm (cy), nucleous (nc) and tight junctions (tj). Arrows indicate the presence of gold particles. The immunogold particles were 10 nm in diameter. Scale bars: 200 nm. Negative controls (NC) for each cell culture treated with the selected MAbs were also performed. No fluorescence or immunogold particles were detected.

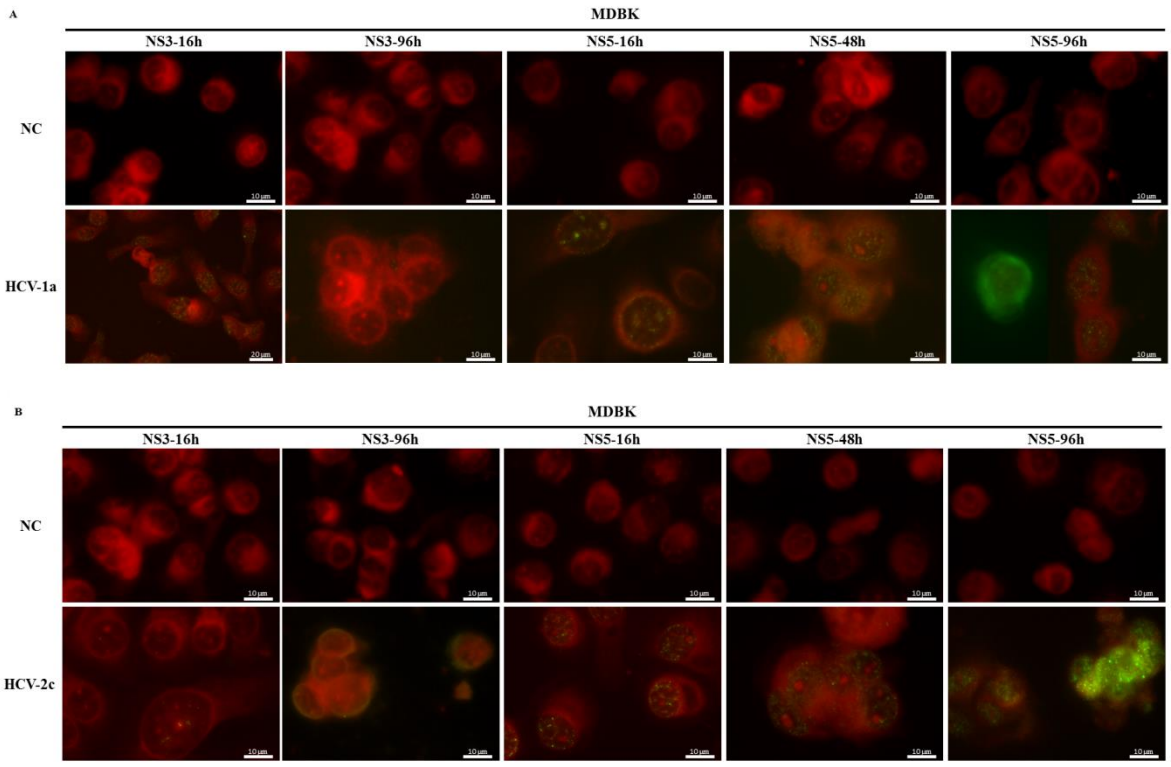


**Figure 3.** Detection of HCV proteins by IFA in MDBK cell cultures infected with HCV-1a and HCV-2c. a, IFA using MAbs anti-HCV NS3 (16 and 48h) and anti-HCV NS5 (16, 48 and 96h) from harvest cells infected with HCV-1a. Scale bars: 10 and 20μm (only HCV-1a NS3-16h). b, IFA using the same MAbs and at the same time points from harvest cells infected with HCV-2c. Scale bars: 10μm. Negative controls (NC) treated with the selected MAbs were also performed and no fluorescence was detected.

# CHAPTER 5

# GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

This dissertation contains one international patent application, two published papers in international journals and two preliminary studies that will be improved for publication in international journals. A detailed discussion and conclusions of each related experimental work is presented in previous chapters of this thesis, relatively to each study. A general discussion and conclusions of the studies carried out are presented in this chapter as well as some future perspectives.

## 5.1 - General Discussion

Hepatitis C is a human contagious liver disease that results from the infection with the HCV, a positive RNA virus, which is characterized by a +ssRNA genome that belongs to the *Flaviviridae* family (1). To date, the HCV origin remains unclear and the HCV life cycle and pathogenesis are not enlightened processes due to the absence of HCV efficient cell cultures systems or animals models. However recently, several studies reporting homologs of HCV detected in small wild animals, rodents and bats, and in domesticated animals, dogs and horses were described (2-4). The discovery of these HCV-like viruses in animals may contribute to the understanding of the HCV origins in humans, and for instance for studies related to the virus pathogenesis or immune responses.

Partially, our investigation, aimed to search for significant natural viruses reservoirs associated to animal or human diseases as explained in the investigation hypothesis of this work as described above, led us to understand the role that the European rabbit (*O. cuniculus*) and European hare (*L. europaeus*) could play as natural reservoirs of *Flaviviridae* and more specifically precursors of HCV-like viruses in this dissertation. Moreover, molecular biology methods such as PCR, sequencing, proteomic, cell cultures and immune screenings methods, as well as bioinformatics tools were procedures aimed for the accomplishment of this proposal.

Therefore, the first chapter of this dissertation includes the state of the art on cell foreign DNA integration, endogenous viral elements, positive RNA viruses, the European rabbit and European hare, rabbits as animal model and the HCV history focusing in the taxonomy, virus life cycle, epidemiology, routes of transmission, diagnosis, therapy, cell cultures systems approaches and proposed animal models.

The second chapter focuses in the study hypothesis that led to this dissertation approaches as well as for the aims of the study.

The third chapter describes the detection and characterization of HCV homologous fragments present in the European rabbit and European hare genome. Preliminary work on the detection of endogenous HCV homolog fragments in the European rabbit and European hare that showed the capacity to infect and autonomously replicate in permissive bovine cell cultures, MDBK epithelial cell line and BT cells respectively was performed. Additionally, total DNA that was extracted from rabbit and hare liver samples and selected synthesized nucleotides or peptides homologous to HCV, were demonstrated to also have the ability to infect and autonomously replicate in MDBK cell cultures. A human wild type HCV-1a isolate was also shown to passively infect and replicate in MDBK cells. This preliminary work led to the application of an international patent (5), as the criterions for a patent application and publication must obligatory anticipate other publications related to the study work. The application for a patent was submitted and published, providing the methods and tools that can contribute for the identification of novel antivirals, vaccine production, new diagnostic tools as well as a better understanding of the HCV life cycle, its origin and diversity, the pathogenesis mechanisms of the virus using the tested bovine cells as cell models. Unfortunately the patent application could not enter the national, European or international phase classification, due to the comments by the written opinion of the international searching authorities (5).

Following the patent application, a manuscript describing that endogenous HCV homolog fragments in European rabbit and hare genomes replicate in cell cultures was published (6). Specifically, it was described that endogenous homologous DNA fragments coding for HCV core, envelope glycoprotein's E1 and E2, protease NS2-3, serine protease NS3, NS4A, NS5A and NS5B specific proteins present in the animal genomes were able to internalize and replicate in MDBK and BT cell cultures, although with no virus-like particles detection or visualization in the analyzed samples (6). As discussed in this publication and above in this thesis, endogenous flaviviruses have previously been reported in the genomes of mosquitoes (7, 8) without determined complete putative genomic structures and EVEs have also been identified and demonstrated in animal genomes (9). Additionally, to understand if the described endogenous homologous HCV fragments in the rabbit and hare would be able to produce entire viral particles with infectious proprieties in the MDBK cell line became a requisite, and it was proven that they

were able, when we demonstrated that HCV-like viruses are produced in MDBK cells, after their inoculation with rabbit and hare DNA extracted from animals livers homogenates samples (10). Moreover, the phylogenetic analysis revealed the presence of viruses genetically similar to HCV, designated rabbit (RHCV) and hare (HHCV) HCV-like viruses, and that RHCV was closely related to the HCV-1a/HCV-1b genotypes and HHCV was more closely related to the HCV-1b genotype (10). As previously discussed in this thesis, we proved that the rabbit and the hare have small EVEs homologous to all HCV genetic specific regions that, when exposed to the MDBK cells (potential suitable biological substrate) are able to internalize the cells and initiate its own replication using the host cell machinery to produce entire HCV-like particles. The previously described functional pathways of ssRNA+ viruses genomes such as, the virion (genomic) RNA is the same sense as mRNA and therefore can function as mRNA that can be immediately translated by host cell machinery upon infection, working the genomic RNA as template for viral RNA replication (11, 12) strongly elucidated us in the understanding and in the viability of all this process to occur.

Finally, a slight improve was achieved for the study on the replication of HCV homologous synthetic fragments in MDBK cells and is described. We investigated if synthetic fragments with total homology between core, E2 and NS4B HCV proteins and the European rabbit genome have the capacity to internalize and replicate in naïve MDBK cell line. This hypothesis was proven by the detection of virus RNA titers of ~3-5 log RNA copies $ml^{-1}$ were detected in tested samples by qRT-PCR analysis. However, more research including these and others synthetic fragments as well as others methodologies must be performed to further confirm this data and the related host-MDBK cell line factors should also be investigated. We will improve this study to accomplish its publication in an international journal. The overall findings prompt us to state that all of the aimed objectives proposed for this dissertation were accomplished.

The fourth chapter of this thesis, the last related for the performed experimental work, and that was not included in the aims of this study but was essential to the patent submission, is related to the hypothesis that bovine cell cultures, MDBK cell line and BT primary cells, could be used as cell models for the wild type HCV replication using patients infected sera, HCV uncharacterized (HCVuc), HCV-1a and HCV-2c genotypes. For BT and MDBK cell cultures infected

with HCVuc, HCV RNA titers ranging from $10^3$-$10^5$ and from $10^3$-$10^6$ IU/ml respectively were detected. Moreover, HCV RNA titers ranging from $10^3$-$10^4$ IU/ml and $10^4$ IU/ml were determined in MDBK cells inoculated with genotypes -1a and -2c respectively. HCV specific immunostaining was also detected in both cell cultures inoculated with the tested samples by immunofluorescence and/or immunogold electron microscopy methods. Immunogold particles were detected in different cell organelles such as nucleous, mitochondria, cytoplasm and tight junctions accordingly with reports previously described for HCV replication in cell cultures (13, 14).

The overall acquired data and the above described presence of essential cell receptors or coreceptors for HCV entry in bovine tissues led us to suggest the bovine cell cultures as cell models for HCV genotypes infection and replication, specially the MDBK cells as they are a cell line culture. The MDBK cells could be a new efficient *in vitro* model for studies of HCV pathogenesis, HCV life cycle, development of new effective antivirals and probably a vaccine production.

## 5.2 - Conclusions

At the beginning of the present thesis we proposed to study the genomic integration of HCV homologous fragments in the European rabbit and European hare, and the possibility for its detection and characterization. We successfully detected HCV homologous fragments in the European rabbit and European hare and were able to further characterize them.

Additionally, we were able to suggest bovine cell cultures as cell culture models for wild type HCV viruses and we showed that MDBK cell line could be a model for wild type HCV genotypes infection and replication. Therefore, the main conclusions of this thesis are:

- Endogenous HCV homolog fragments coding for such as HCV core, envelope glycoprotein's E1 and E2, protease NS2-3, serine protease NS3, NS4A, NS5A and NS5B specific proteins were demonstrated in European rabbit and hare genomes.
- The endogenous HCV homolog fragments are able to replicate in bovine cell cultures, MDBK cell line and BT primary cell cultures.
- Hepatitis C-like viruses are produced in MDBK cell cultures after inoculation of rabbit and hare genomic DNA and named RHCV and HHCV respectively.

- The produced RHCV is closely related to the HCV-1a/HCV-1b genotypes and HHCV is more closely related to the HCV-1b genotype.
- To date the produced RHCV and HHCV are the most HCV-like viruses associated to animals that are more closely related to HCV.
- The suggestive replication of synthetic fragments homologs to HCV was demonstrated in MDBK cells.
- Bovine cell cultures, particularly the MDBK cell line could be a considered a cell model for HCV replication.
- The data presented in this dissertation could contribute to understand the HCV origin and for a better understanding of the virus life cycle and pathogenesis, as well as contribute to the discovery of new antivirals and perhaps a vaccine production.

### 5.3 - Future Perspectives

- Improve the study of the synthetic fragments in MDBK cell cultures. Also evaluating their functional mechanisms in the perspective and approach for new antivirals or new antiviral targets.
- Improve the study on the mechanisms of wild type HCV strains replication in MDBK cell cultures.
- Explore the MDBK cell line in the context of the detection and understanding the receptors or coreceptors present in the MDBK cells that make them permissive for these viruses.
- Explore the HCV life cycle in MDBK cells.
- Initiate animal experimental pathogenesis studies using the rabbit as a model: inoculation of rabbits with produced HCV-like viruses, RHCV and HHCV respectively as well as with HCV wild type
- Further explore the rabbit as an animal model for research on HCV.

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# ANNEXES

# POSTERS AND ETHICS COMMITTEE DECLARATION

**1 - Poster 1 - Silva E, Marques S, Thompson G. Hepatitis C Virus Infection and Replication in Bovine Cell Cultures. 3rd World Congress on Virology. Baltimore, USA, 2013**

Virology-2013

OMICS Group Conferences
Accelerating Scientific Discovery

3rd World Congress on Virology

November 20-22, 2013 DoubleTree by Hilton Baltimore-BWI Airport, USA

# Hepatitis C Virus infection and replication in bovine cell cultures

**Eliane Silva, Sara Marques, Gertrude Thompson**

*Universidade do Porto, Portugal*

## Abstract

Hepatitis C virus (HCV) is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. A detailed understanding of the virus life cycle, the development of new antivirals as well as a vaccine is urgent. HCV infects and replicates in cell culture systems like Huh7, Huh7.5, Vero and mosquito cells among others. However, these systems were not reliable when inoculated with the natural virus or using them in antiviral or complete virus live cycle studies. We recently described that endogenous HCV homolog fragments can efficiently replicate in bovine cell cultures, Madin-Darby bovine kidney (MDBK) epithelial cell line and bovine testis (BT) primary cells, respectively. In this study we show that MDBK and BT cell cultures can sustain HCV infection and replication after inoculation with patients infected sera of HCV-1a and -2c genotypes. The HCV infection and replication in the bovine cell cultures were demonstrated by quantitative Real Time-PCR, immunofluorescence assay and immunogold electron microscopy using specific monoclonal antibodies for HCV specific proteins. Our results provides the means for a better understanding of the virus pathogenesis and new insights for the *in vitro* screening of new and more efficient HCV antivirals and vaccines development.

## Biography:

Eliane Silva is a technician at the Institute for the Biomedical Sciences Abel Salazar (ICBAS), University of Porto (UP), since 1998. She completed her degree in biochemistry in 2011 from the Faculty of Sciences, UP, and currently she's a PhD student in virology at ICBAS / CIBIO, UP. Sara Marques, DVM, is a Post Doc at the Laboratory of Infectious Diseases in ICBAS / CIBIO, UP. She has obtained her PhD in Veterinary Sciences in 2011 in ICBAS, UP. Gertrude Thompson, DVM, MSc, PhD, is Aggregated Associate Professor of Microbiology and Infectious Diseases at ICBAS, UP. Their main interests are Infectious and Emerging Diseases, Zoonosis, Antimicrobial resistance, Public health, Biotechnology, Virology, Microbiology, Genetics, Proteomics and Microscopy. They have more than 10 papers published together in international indexed journals.

**2 - Poster 2 - Silva E, Osório H, Thompson G. Recovery of HCV-like viruses from naïve MDBK cell line inoculated with rabbit and hare DNA. The Viral Hepatitis Congress 2015, Frankfurt, Germany. Poster Abstracts. J Viral Hep 2015;22:19-49**

JOURNAL OF VIRAL HEPATITIS JVH

*Journal of Viral Hepatitis,* 2015, **22** (Suppl. S3), 19–49 doi:10.1111/jvh.12441

# Poster Abstracts



**Table 1** - SVR12 rates by patient subgroups

| SVR12, % (n/N) | 12 week naïve | 12 week exp'd | 8 week naïve | SV12, % (n/N) | 12 week naïve | 12 week exp'd | 8 week naïve |
|---|---|---|---|---|---|---|---|
| All patients | 97 (98/101) | 98 (51/52) | 76 (38/50) | Male | 98 (90/92) | 98 (42/43) | 79 (33/42) |
| [95% CI] | [89.8, 99.2] | [88.0, 99.9] | [59.7, 87.6] | Female | 89 (8/9) | 100 (9/9) | 63 (5/8) |
| GT 1 | **96 (80/83)**[a] | 98 (43/44) | 76 (31/41) | Age 65 year | 97 (93/96) | 98 (48/49) | 77 (36/47) |
| GT 1a | 96 (68/71) | 97 (32/33) | 80 (29/35) | Age ≥65 year | 100 (5/5) | 100 (3/3) | 67 (2/3) |
| GT 1b | 100 (12/12) | 100 (11/11) | 50 (3/6) | White race | 96 (63/66) | 100 (31/31) | 71 (20/28) |
| GT 2 | 100 (11/11) | 100 (2/2) | 83 (5/6) | Black race | 100 (30/30) | 95 (19/20) | 79 (15/19) |
| GT 3 | 100 (6/6) | 100 (4/4) | 67 (2/3) | *IL28B* CC | 100 (28/28) | 100 (13/13) | 69 (9/13) |
| GT 4 | 100 (1/1) | 100 (2/2) | - | *IL28B* Non-CC | 96 (70/73) | 97 (38/39) | 78 (29/37) |
| BL HCV RNA <6 million IU/mL | 97 (56/58) | 100 (33/33) | 79 (27/34) | PI cART | 98 (46/47) | 96 (22/23) | 72 (21/29) |
| | | | | NNRTI cART | 100 (28/28) | 100 (12/12) | 80 (8/10) |
| BL HCV RNA ≥6 million IU/mL | 98 (42/43) | 95 (18/19) | 69 (11/16) | Other cART | 92 (23/25) | 100 (16/16) | 78 (7/9) |
| | | | | BL CD4 200 c/mm$^3$ | 100 (4/4) | - | 100 (1/1) |
| No cirrhosis | 98 (88/90) | 100 (34/34) | 77 (34/44) | BL CD4 200–499 c/mm$^3$ | 98 (41/42) | 100 (12/12) | 71 (15/21) |
| Cirrhosis | 89 (8/9) | 93 (14/15) | 60 (3/5) | BL CD4 ≥500 c/mm$^3$ | 96 (53/55) | 100 (39/39) | 79 (22/28) |

BL, baseline; c, cells; cART, combination antiretroviral therapy; CI, confidence interval; GT, genotype; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor
[a]Primary endpoint.

inhibitors). A total of 98% of patients completed study treatment. Among GT-1 patients, SVR12 was achieved by 96% of naïve and 98% of experienced patients after 12 weeks of DCV+SOF and by 76% of naïve patients after 8 weeks; SVR12 rates for non-GT-1 patients in these groups were 100%, 100%, and 78%, respectively. SVR12 was similar in patients with or without cirrhosis and across other demographic and disease subgroups (Table 1). There were no HCV virologic breakthroughs, and HIV control was not compromised throughout the study period. Post-treatment HCV relapse occurred in 1–2% of patients in the 12-week treatment groups and 20% in the 8-week group. There were no treatment-related serious AEs or discontinuations for AEs.

**CONCLUSIONS:** Treatment of HIV-HCV coinfected patients with DCV+SOF once daily for 12 weeks resulted in an overall 97% SVR12, and was well tolerated. DCV+SOF was effective in cirrhotics, in other demographic and disease categories, and across a broad range of cART regimens without compromising HIV virologic control.

## Molecular biology and characterisation

### P21

### Recovery of HCV-like viruses from naïve MDBK cell line inoculated with rabbit and hare DNA

E Silva[1], H Osório[2] and G Thompson[1] [1]*ICBAS/CIBIO/InBIO – University of Porto, Porto, Portugal,* [2]*I3S, IPATIMUP, FMUP – University of Porto, Porto, Portugal*

**PURPOSE OF THE STUDY:** Hepatitis C virus (HCV) contains an ssRNA+ genome which, upon virus entry and uncoating, functions as mRNAs and thus can be directly translated into proteins by host cell machinery (1,2). We recently described the presence of endogenous HCV homolog fragments in wild/domestic rabbits and hare genomes and their capacity to replicate in Mardin-Darby Bovine Kidney (MDBK) cell cultures (3). To understand if these small endogenous fragments were able to produce infectious entire virus particles in this same cell line was the purpose of the study.

**METHOD:** DNA extracts from liver homogenates of a domestic rabbit and a hare were subjected to RNAse treatment and directly inoculated in naïve MDBK cells. Their capacity to generate entire HCV-like virus particles and infectivity were evaluated by immunogold electron microscopy (IEM) with monoclonal antibodies for the NS5 and E2 HCV specific proteins, quantitative Real Time-RT-PCR (qRT-PCR) and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF-MS/MS) mass spectrometry. A phylogenetic analysis of a final consensus constructed sequence was performed for each tested sample.

**SUMMARY OF RESULTS:** Specific immunostaining was observed in cell suspensions of passages of inoculated MDBK cell flasks using mouse monoclonal antibodies anti-HCV NS5 (P4 and P7) and anti-HCV E2 (P4) proteins, by IEM. HCV RNA titers were measured by qRT-PCR of inoculated cells at passages P1 to P7, with ranges of 3.75–5.83 log RNA copies/ml and 4.36–5.91 log RNA copies/ml detected from rabbit and hare DNA samples respectively. By MALDI-TOF/TOF-MS/MS a total of 2338 peptide sequences, such as F, Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B HCV were identified with significant protein scores (P.S.) (P.S. >64 are significant, $p < 0.05$). Of these, 1694 and 644 were from the rabbit and hare DNA samples respectively.

 

The phylogenetic analysis of a constructed consensus sequences of the HCV-like viruses from rabbit (RHCV) and hare (HHCV), using the NJ and the ML methods, revealed that RHCV is more closely related to HCV-1a/HCV-1b genotypes and HHCV to HCV-1b genotype.

**CONCLUSION:** RHCV and HHCV HCV-like particles are produced in the MDBK cell line, suggesting that the small fragments present in the genomic DNA of the rabbit and hare can, after internalizing the MDBK cells, initiate replication and together with the cell machinery generate novel HCV-like viruses.

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## P22

### An outbreak of acute HCV infection with genotype 1a in a Haemodialysis Unit in Kocaeli, Turkey

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**OBJECTIVE:** To analyse an outbreak of acute HCV infection in a private Haemodialysis Unit. The genotype of chronic HCV infection is over 90% genotype 1 (distribution of subgroup analysis approximately 75% 1b, 25% 1a) in Turkey.

**MATERIALS AND METHODS:** There were 125 patients treated in a private Haemodialysis Unit. They have known 12 HCV infections before the outbreak. A separate machine was used for these patients. Then began new HCV infection cases from July 2013 that not infected before. A total of 26 new HCV infections in serial were identified in approximately 5 months from the same unit. The diagnosis was based on ALT elevations, anti-HCV detection and HCV-RNA detection. Other virological tools including HCV genotype determination and NS 3 gene phylogenetic tree analysis of the acute hepatitis C epidemic were also used to tailor the epidemic nature. HCV genotype/subtypes were identified by phylogenetic analysis of NS3 sequences (codon 27–181 of protease domain). Nucleotide sequences were compared consensus HCV sequences from Strain H77, D90208, HPCPLYPRE, HPCCGAA, HPCJCG, HPCHUMR, HPCCGS and AY051292. Phylogenetic comparisons were performed using neighbour-joining method with the CLC Sequence Viewer 6.9.1 (CLC bio A/S, Denmark). All the personnel who worked there were tested for HCV infection. We took over 100 samples from different machines, solutions that used in the unit.

**RESULTS:** We identified 26 (10 female and 16 male) with acute hepatitis C infection between July and November 2013. The mean HCV RNA were 7217 432 IU/mL. All of them were known anti-HCV negative before this epidemic. The known HCV positive patients of the center were all genotype 1b. But in this epidemic we found another responsible strain. Phylogenic analysis identified one distinct HCV group and genotype 1a. The index case-patient is not known yet. No multidose medication vials or material was shared between patients. The infected patients had used different dialysis machines. The working personnel were found all negative for HCV infection.

**CONCLUSION:** During this outbreak, HCV transmission was mainly with a new patient with acute nonsymptomatic infection to another patient via healthcare workers' hands. We could not find another possible source in the unit.

## P23

### Bioinformatic analysis of codon usage and phylogenetic relationships of different genotypes of hepatitis C virus

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**BACKGROUND:** Hepatitis C viral infection has six major genotypes. The purpose of this study was to investigate phylogenetically different genotypes of hepatitis C virus and amino acid codon usage in the structure of the virus proteins to discover new methods of treatment regimes.

**MATERIALS AND METHODS:** Codon usage of the six genotypes of HCV nucleotide sequence was investigated through the online application available on the website of Gene Infinity. Also, phylogenetic analysis and evolutionary relationship of HCV genotypes were analyzed using software MEGA 4.

**RESULTS:** In the first group genotypes 1 and 5 (74.02%) and in the second group genotypes 2 and 6 (72.43%) have the most similarity on codon usage. Unlike the results of similarity of codon usage, phylogenetic analysis study showed the most closely resembles and correlation between genotype 1 and 4.

**CONCLUSION:** Genotypes 1 and 4 have the remarkable similarity of genome sequences and proteins, but in terms of preferred codons for genes expression have the greatest difference. More and additionally studies are needed to confirm the results and select the best approach for treatment of these genotypes based of preferred codons.

## 3 - Declaration approved by the Ethics Committee of the Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), University of Porto, Porto, Portugal (CETI; Projeto Nº 086/2014) for the use of the human sera samples

Comissão de Ética

U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

***Parecer da Comissão de Ética do ICBAS-UP***

**PROJETO Nº 086/2014**

**Título:** Genomic integration of Hepatitis c virus fragments in the european rabbit and *lepus europaeus*: detection and characterization.
**Investigador Responsável:** Gertrude Thompson
**Outros investigadores:** Eliane Pimenta da Silva

A Comissão de Ética do ICBAS-UP reuniu dia 13 de fevereiro de 2015 no edifício do ICBAS - Sala de reuniões do Departamento de Ciências do Comportamento, na presença de Liliana de Sousa, Manuel Vilanova, Margarida Araújo, Maria Antónia Gonçalves e Mário Paulo Maia.

Dada a natureza do estudo (*in vitro*) e das amostras humanas utilizadas a CETI não tem nada a opor ao trabalho realizado no estudo "*Naïve Madin-Darby Bovine Kidney Cell Line Sustains the Infection and Efficient Replication of Hepatitis C Virus Genotype 1a*".

Com os melhores cumprimentos,

Pela Comissão de Ética do ICBAS-UP,

Prof. Doutora Liliana de Sousa (presidente)

*To whom it may concern,*

*The above project is in accordance with the Portuguese law and the ICBAS-UP Ethics Committee criteria.*