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Review

Flavivirus reverse genetic systems, construction techniques and applications: A historical perspective

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ABSTRACT

The study of flaviviruses, which cause some of the most important emerging tropical and sub-tropical human arbovirus diseases, has greatly benefited from the use of reverse genetic systems since its first development for yellow fever virus in 1989. Reverse genetics technology has completely revolutionized the study of these viruses, making it possible to manipulate their genomes and evaluate the direct effects of these changes on their biology and pathogenesis. The most commonly used reverse genetics system is the infectious clone technology. Whilst flavivirus infectious clones provide a powerful tool, their construction as full-length cDNA molecules in bacterial vectors can be problematic, laborious and time consuming, because they are often unstable, contain unwanted induced substitutions and may be toxic for bacteria due to viral protein expression. The incredible technological advances that have been made during the past 30 years, such as the use of PCR or new sequencing methods, have allowed the development of new approaches to improve preexisting systems or elaborate new strategies that overcome these problems. This review summarizes the evolution and major technical breakthroughs in the development of flavivirus reverse genetics technologies and their application to the further understanding and control of these viruses and their diseases.

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Contents

1. Introduction . . . 68
2. From past to present . . . 68
3. Flavivirus reverse genetics systems and construction strategies . . . 70
   3.1. Infectious clones . . . 71
      3.1.1. Production of cDNA . . . 71
      3.1.2. Choice of host for cloning the cDNA . . . 72
   3.2. Bacteria-free approaches . . . 74
      3.2.1. Long PCR . . . 74
      3.2.2. Circular polymerase extension reaction and Gibson assembly . . . 74
      3.2.3. Infectious subgenomic amplicons . . . 74
4. Impacts/utilisations . . . 75
   4.1. Reverse genetics methods and clonality of viral populations . . . 75
   4.2. Reverse genetics methods as research tools . . . 75
      4.2.1. Studying point mutations . . . 75
      4.2.2. Host/pathogen interactions . . . 76
      4.2.3. Viral proteins . . . 78
      4.2.4. Protein modifications . . . 78
      4.2.5. Cyclization sequences and RNA secondary structures . . . 79
   4.3. Reverse genetics methods as therapeutic tools . . . 80

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4.3.1. Chimeric vaccines . . . 80
4.3.2. Antiviral drugs . . . 80
5. Conclusions and perspectives . . . 81
References . . . 81

1. Introduction

According to the IXth Report of the International Committee on Taxonomy of Viruses (King et al., 2011), the genus *Flavivirus* (family *Flaviviridae*) contains 53 species of which 40 are known to cause human disease including dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV). These viruses cause millions of human infections each year ranging from mild febrile symptoms to fatal hemorrhagic/neurologic disease (Gubler, 2002; Guzman et al., 2010; Halstead and Thomas, 2010; Hayes et al., 2005; Monath, 2008). During recent decades, the dramatic increase in the movement of humans, animals and commodities via modern transportation, unplanned urbanization in tropical developing countries and the lack of adequate public health infrastructures have enabled these pathogens to expand their geographic ranges to cause major epidemics (Artsob et al., 2009; Duffy et al., 2009; Gould and Solomon, 2008; Gubler, 2004).

Flaviviruses are small (~50 nm), enveloped, single-strand positive-sense RNA viruses. Their genomes vary between 10.5 and 11 kb and contain a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs) of approximately 100 nucleotides and 500 nucleotides respectively that form specific secondary structures required for genome replication and protein translation (Gritsun and Gould, 2007; Lindenbach et al., 2007; Strauss and Strauss, 1988). Translation of the ORF leads to synthesis of a polyprotein that is cleaved by host and viral proteases to release structural and non-structural viral proteins (Harris et al., 2006). The structural proteins (C, prM, and E) are located in the N-terminal portion of the polyprotein and the non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) in the remaining C-terminal portion of the polyprotein (Lindenbach et al., 2007).

The 5′ end of the genome possesses a type I cap (m7GpppAmp) and the 3′ end of the genome lacks a 3′ polyadenylate tail (Dong et al., 2012). The flaviviruses also contain a unique, subgenomic, small (0.3–0.5 kb) non-coding RNA present in the 3′ UTR of the viral RNA, which is thought to be essential for virus replication in cells and to modulate pathogenicity in animals (Pijlman et al., 2008; Roby et al., 2014). More recently, a ribosomal frameshift site was identified in the JEV serogroup that gives rise to the NS1′ protein, an elongated form of NS1 protein (Firth and Atkins, 2009; Melian et al., 2010; Ye et al., 2012).

It has been known for years that the RNA of many positive-strand RNA viruses, including flaviviruses, is infectious when transfected into susceptible tissue culture cells (Baltimore, 1971a,b; Boyer and Haenni, 1994). The first reports of the infectivity of animal virus nucleic acids were made in 1957 by Colter et al. for Mengovirus, Poliovirus and WNV and by Wecker and Schafer for *Eastern equine encephalitis virus* (Colter et al., 1957; Wecker and Schafer, 1957). This principle has been exploited as molecular techniques have improved. A bacterial DNA plasmid clone containing the full-length complementary DNA (cDNA) of a positive-strand RNA virus and a suitable promoter, such as T7 can be used to produce infectious genomic viral RNA by *in vitro* transcription. These DNA clones enable the introduction of mutations into viral genomes and the consequent study of the resulting phenotype (Ruggli and Rice, 1999). This powerful molecular tool is frequently referred to as reverse genetics.

Infectious DNA clones have been obtained and used to study important virus properties such as virulence/attenuation, cell penetration, replication, host range and functions of coding or non-coding genomic regions. However, their construction is often difficult, involving cloning of many subgenomic cDNA fragments. In spite of recent improvements afforded by novel molecular techniques, the process is still laborious and many difficulties remain including instability and toxicity of some viral sequences in bacterial hosts (Gritsun and Gould, 1995; Rice et al., 1989; Ruggli and Rice, 1999).

The aim of this review is to describe the important historical steps in the development of flavivirus infectious clones, to summarize the different reverse genetics strategies that are currently used and to assess the impact of this technology on our understanding and control of these viruses and their diseases.

2. From past to present

In 1981, Racaniello et al. showed for the first time that the full length cDNA of a eukaryotic positive stranded RNA virus, the poliovirus, can generate infectious virus in mammalian cells directly after transfection (Racaniello and Baltimore, 1981b). This was achieved by cloning the complete genome as 3 separate segments (Racaniello and Baltimore, 1981a) and then producing a complete cDNA copy of the genome in a bacterial plasmid. Cultured mammalian cells transfected with this plasmid produced infectious poliovirus.

Subsequently the development of the polymerase chain reaction (PCR) in 1986 marked a turning point in infectious clone technology as it completely simplified the methodology for cloning; it was no longer necessary to extract large quantities of viral RNA from infectious cultures for reverse transcription (Mullis et al., 1986). With the development of *in vitro* transcription systems and the use of bacteriophage promoters (SP6, T7), it also became possible to synthesize *in vitro* full length viral RNA with a much higher efficiency when compared with cDNA transcription in the cell. Furthermore, development of more efficient transfection methodologies, such as the use of cationic liposomes (Malone et al., 1989) and electroporation, improved the efficiency of RNA transfection of cultured cells. Thus, the basic methodology for reverse genetics technology was established.

The first reverse genetics model for the study of flaviviruses was published in 1989 (Rice et al., 1989). As for poliovirus, the complete genome sequence of the YFV 17D vaccine strain was determined (Rice et al., 1985) resulting in a profound reorganization of the classification of arboviruses (flaviviruses and alphaviruses were assigned to different families using data from their genome organization). During this developmental work, cloning the complete genome into bacterial plasmids proved to be impossible due to instability of the construct in *Escherichia coli*. This difficulty was circumvented by cloning two segments of the genome in separate plasmids. They were then assembled by *in vitro* ligation and amplified by *in vitro* transcription before transfection into cells (Rice et al., 1989).

Subsequently, reverse genetics models were described for a number of other flaviviruses, namely JEV (Sumiyoshi et al., 1992), DENV (Gualano et al., 1998; Kapoor et al., 1995; Kinney et al., 1997; Lai et al., 1991; Polo et al., 1997; Puri et al., 2000), Kunjin

virus (KUNV) (Khromykh and Westaway, 1994), Murray valley virus (MVV) (Hurrelbrink et al., 1999), TBEV (Mandl et al., 1997) and WNV (Yamshchikov et al., 2001b). As for the YFV 17D vaccine strain, it was not always possible to obtain full-length cDNA cloned in a single plasmid (e.g., for JEV and DENV2 (Kapoor et al., 1995; Sumiyoshi et al., 1992). Thus, two clones containing roughly half of the genomic cDNA were generally used. At this stage of the development period many steps, including sub-cloning, correction of mutations introduced by error-prone DNA polymerase, exchanging plasmid backbone, were required often involving several years additional work.

From the late 1980s, the race to obtain reverse genetics models for flaviviruses took advantage of many technological advances and new strategies were described to circumvent the difficulties (Edmonds et al., 2013; Gritsun and Gould, 1995; Khromykh et al., 2001; Rice et al., 1989; Ruggli and Rice, 1999). For example, the development of the PCR as well as the reverse transcription PCR (RT-PCR) and in particular their improvements in terms of fidelity, processivity and speed, was a paradigm shift (Barnes, 1994; Cheng et al., 1994; Gritsun and Gould, 1995, 1998). These improvements encouraged virologists to develop a variety of reverse genetic systems. However, commercial kits were not available; consequently scientists had to develop their own protocols. The development and availability of reliable commercial molecular biology kits has significantly accelerated virological research capability.

During this developmental period a variety of methods were reported to achieve similar objectives. For example, the use of long high-fidelity PCR combined with long RT provided a new approach for the construction of recombinant viruses (Gritsun and Gould, 1995, 1998; Gritsun et al., 2001). The pioneering work of Gritsun and Gould (1995) significantly improved this method by using a combination of primer sets and optimizing their concentrations (Gritsun and Gould, 1995). They produced two long overlapping PCR products (5.2 and 5.7 kb) by RT-PCR, ligated and transcribed them into full-length viral RNA. Using this principle, infectious full-length RNA transcripts were realizable even for situations in which the virus genome could not easily be cloned in bacteria. Moreover, it avoided the need for virus purification and shortened the time to produce engineered virus from years to days. However, despite the success in generation of infectious transcripts from long RT-PCR amplicons without cloning, a stable full-length clone of virus genomes is still a valuable genetic resource for DNA (and ultimately) RNA manipulation.

Improved sequencing methods were another essential requirement for the generation of reverse genetics models. Sanger's method (dideoxy or chain-termination) was the traditional approach to DNA sequencing (Sanger et al., 1977). Initially, the methods were completely manual and used radioactive ladders displayed on huge acrylamide gels. Subsequently, the radioisotopes were replaced using four different fluorescent dyes in the sequencing reactions (Smith et al., 1985). This improvement rapidly led to the development of the first automated fluorescence DNA sequencer (Ansorge et al., 1986, 1987; Brumbaugh et al., 1988; Hood et al., 1987; Hunkapiller, 1991; Hunkapiller et al., 1991; Prober et al., 1987; Smith, 1986). The next breakthrough was made with the description of the first high electric field separations of DNA sequencing fragments using capillary electrophoresis in 1990 (Swerdlow and Gesteland, 1990) and the first adaptation to display the results as an array (Zagursky and McCormick, 1990). From this adaptation, the first commercial automated DNA sequencer that used capillary electrophoresis rather than a slab gel, became available in 1996.

With these new automated machines the accumulation of DNA sequence data accelerated rapidly. The early pioneer of sequence databases was Margaret Dayhoff who had previously established a protein sequence database and published the first collection of nucleotide sequence information in 1981 (Dayhoff et al., 1981). Shortly thereafter, GenBank was created in 1982 by the National Institute of Health, USA. GenBank provided a timely, centralized, accessible repository for genetic sequences (Bilofsky et al., 1986). In parallel with the growth of databases, methods to prepare robust sequence alignments for detailed comparison soon became the rate-limiting step in the analysis of sequence data. The development of rapid search programs such as FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990) made it practical to identify genes in a new sequence by comparison with sequences already in the databases. Bioinformatics which was in its infancy suddenly became essential for the interpretation of these sequence data and for the generation of testable hypotheses arising from the data. The first sequence analysis software then began to appear such as MEGA which offered the possibility of producing phylogenetic trees from nucleotide and amino acid sequence alignments (Kumar et al., 1994). The production of software for analysis of sequence data is now a major growth industry, with new and improved methods appearing frequently.

The automated Sanger DNA sequencing method is recognized as the "first-generation" technology, and newer DNA sequencing methods are referred to as "next-generation" sequencing (NGS). These new technologies utilize various strategies that rely on

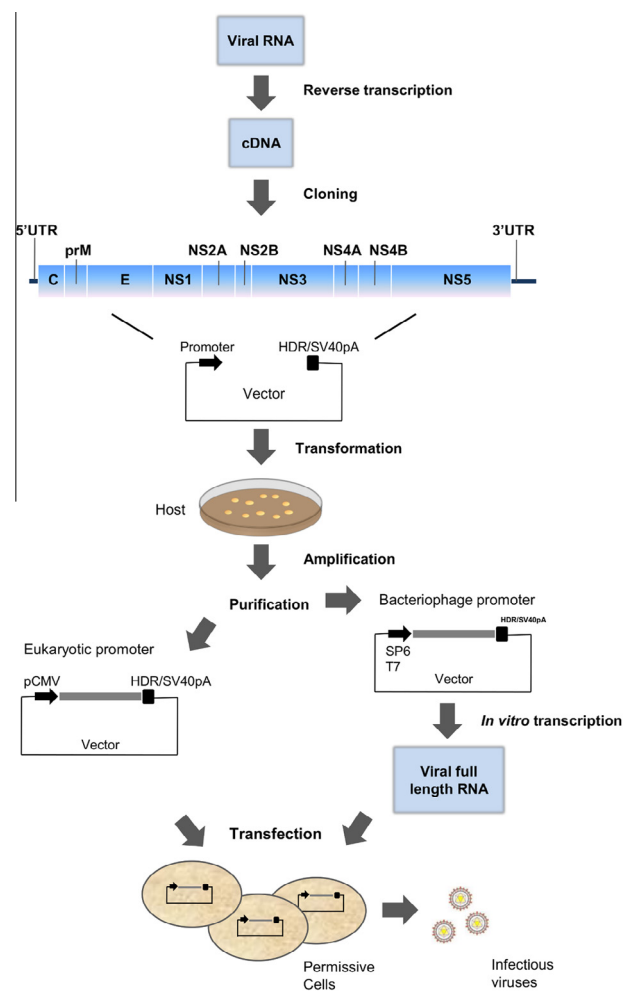

Fig. 1. Generic procedure for infectious clone construction. A double stranded cDNA copy of an RNA virus genome is stably incorporated into a vector and amplified into a host. After purification of the construction, infectious viruses are obtained either by direct transfection of permissive cells when an eukaryotic promoter is used or by transfection of genomic RNA obtained by *in vitro* transcription when a bacteriophage promoter is used.

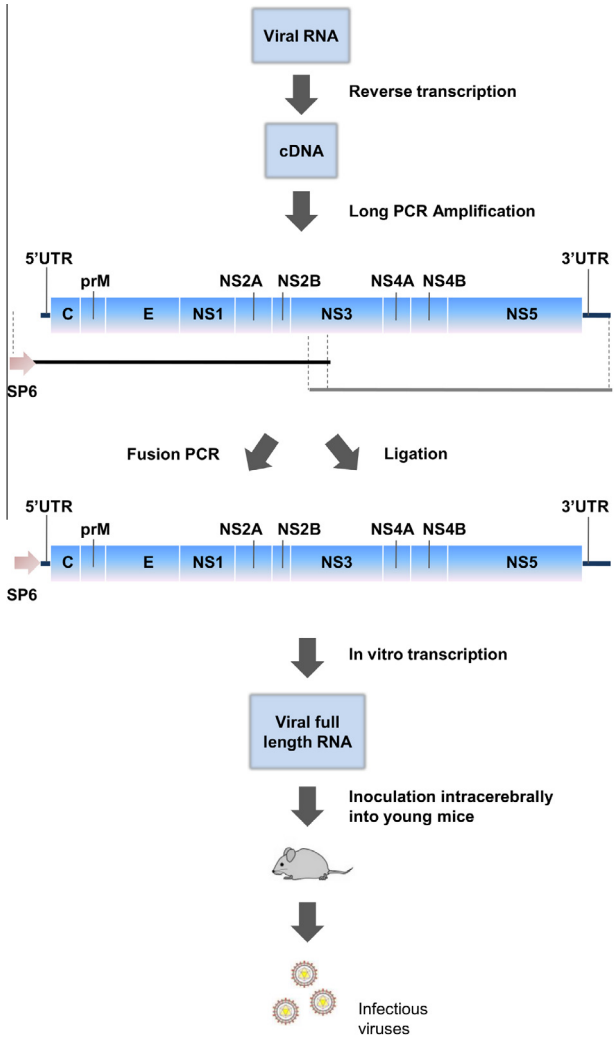

Fig. 2. Bacteria-free approach, generic procedure for production of TBEV using Long PCR. Two Long PCR products were either ligated using restriction enzymes or merged by fusion PCR. After *in vitro* transcription and inoculation of the full-length RNA transcripts intracerebrally into young mice, infectious viruses were recovered.

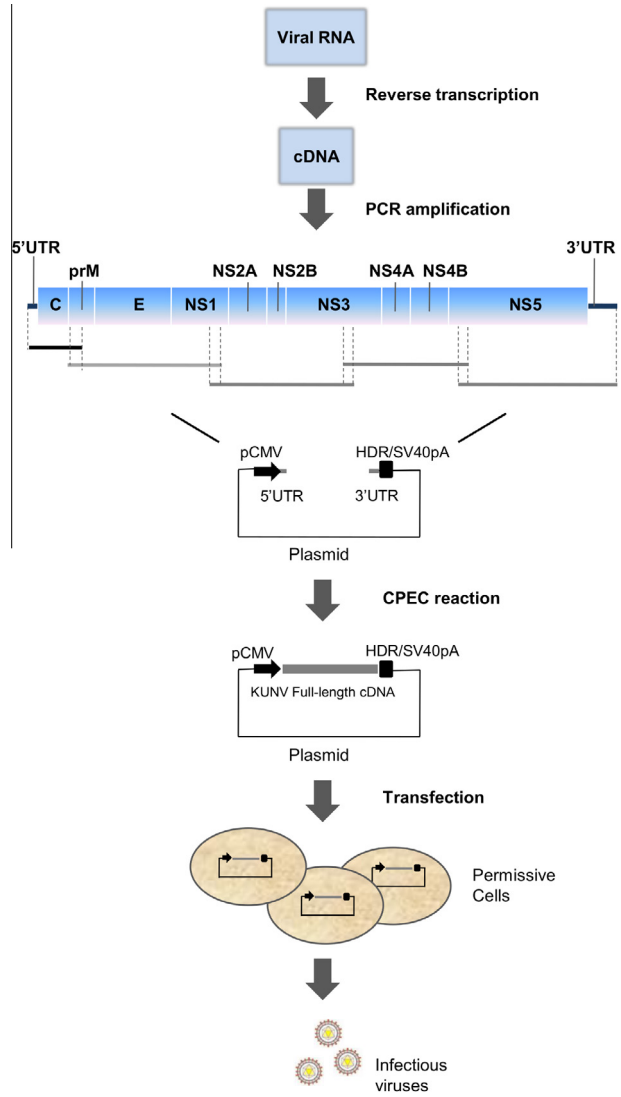

Fig. 3. Bacteria-free approach, generic procedure for production of KUNV using CPEC reaction. Phusion high-fidelity DNA polymerase was used to assemble and incorporate multiple RT-PCR amplicons into a vector. The generated circular product was then transfected directly into competent cells to recover infectious viruses.

different combinations of template preparation, sequencing and imaging, high speed data analysis and throughput with the capacity to produce enormous quantities of sequence data both rapidly and at increasingly lower cost. The first next-generation high-throughput sequencing technology, the 454 FLX pyrosequencing platforms, became available in 2005. By early 2007, Illumina had released the Genome Analyzer, then SOLiD was released (Glenn, 2011). This technological aspect of sequencing and analysis is expanding rapidly with novel and improved platforms continuously being developed and released. The most recent examples include Heliscope and Ion Torrent PGM which became available in 2010. An important consequence of these developments is that complete sequences can now be obtained rapidly regardless of origin, and whether or not they are already known. This rapid progress has totally altered the speed and ease with which reverse genetics systems can be produced and used to study previously inaccessible subjects.

DNA synthesis techniques and technologies are also evolving rapidly to meet these new scientific demands. Entire genomes can now be synthesized and as the demand has increased, *de novo* synthesis has become a cheap alternative from which to obtain cloned cDNA. One of many consequence of these improved methodologies is that it is now technically possible to resuscitate viruses for which no infectious virions exist (Cello et al., 2002; Tumpey et al., 2005) and in theory it should now be possible to create novel viruses synthetically. This approach was first explored by Blight et al. (2000) for the creation of a replication-competent HCV RNA replicon (Blight et al., 2000).

During the early stages of development in the 1980s, a lack of adapted technologies and time constraints were the primary obstacles for generating reverse genetics models. Today, the production of a reverse genetic system is a relatively simple step towards understanding the mechanisms governing the biological functions of viruses.

3. Flavivirus reverse genetics systems and construction strategies

Reverse genetics is defined as the generation of a virus entirely from cDNA (Neumann and Kawaoka, 2004). It is a powerful tool when studying viruses because they are relatively easy to manipulate technically and their short virus replication cycles facilitate

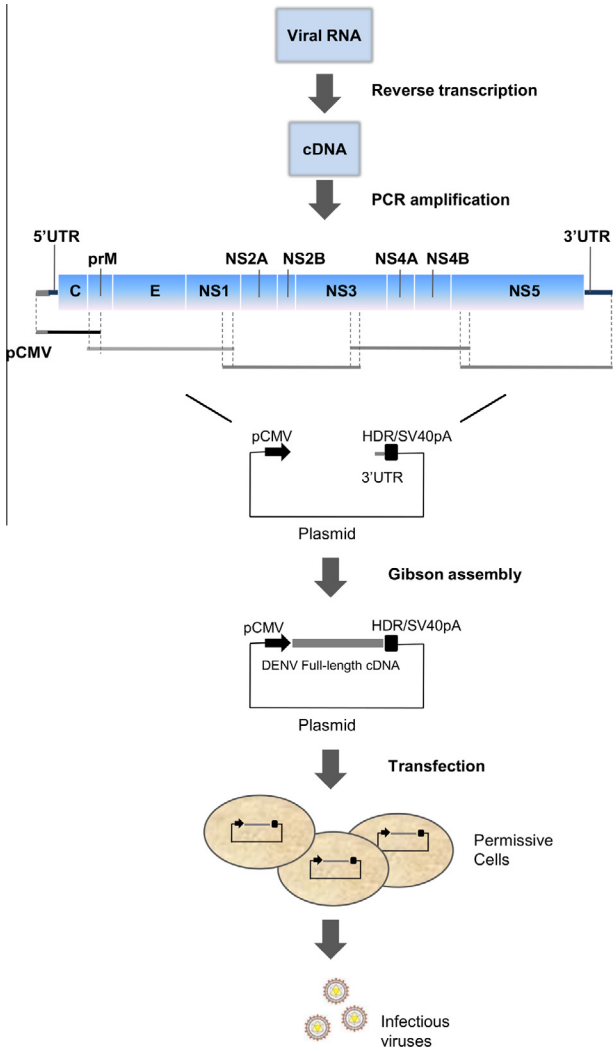

Fig. 4. Bacteria-free approach, generic procedure for production of DENV using Gibson assembly method. Using a combination of three enzymes (exonuclease, DNA polymerase and DNA ligase), multiple RT-PCR amplicons of both virus and vector are assembled during an isothermal and single-reaction. The generated circular product was then transfected directly into competent cells to recover infectious viruses.

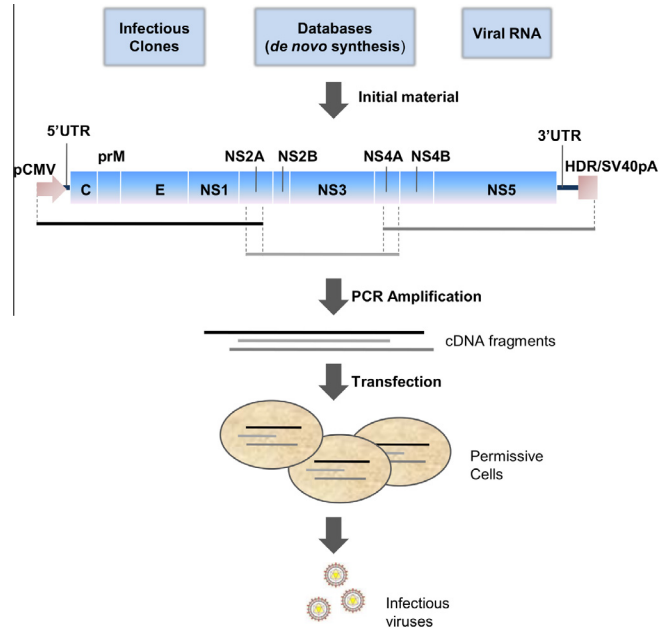

Fig. 5. Bacteria-free approach, generic procedure for production of flaviviruses using the ISA method. The entire viral genome, flanked at the 5′ extremity by the human cytomegalovirus promoter (pCMV) and at the 3′ extremity by the hepatitis delta ribozyme followed by the simian virus 40 polyadenylation signal (HDR/SV40pA), was amplified by PCR in three overlapping cDNA fragments. Direct transfection of PCR products into competent cells enabled the recovery of infectious viruses (successfully applied to DENV, YFV, JEV and TBEV).

rapid phenotypic expression of modified viruses, potentially for use as vaccines or vectors, for analysis of viral genes, non-coding sequences or even for studies of life cycles, pathogenesis or transmission. The most commonly used and powerful reverse genetics system for studies of flaviviruses is known as "infectious clone" (see Fig. 1), but other alternative systems have been developed and are described below (see Figs. 2–5).

3.1. Infectious clones

An infectious clone is a cDNA copy of an RNA virus genome that can be stably incorporated into a vector and from which genomic RNA can be obtained by *in vitro* transcription. Because this RNA is potentially infectious, its transfection into appropriate cells leads to the recovery of infectious virus. To manipulate these viruses genetically, a cDNA copy of the RNA genome is cloned. If required, this cDNA can then be modified by site-directed mutagenesis or *de novo* synthesis, using residual or introduced restriction sites. Appropriate plasmids, cosmids, bacteria or a variety of yeasts, can be used as vectors to clone this cDNA. The presence of a DNA dependant RNA polymerase promoter at the 5′ end of the sequence is required to enable efficient generation of viral RNA either by *in vitro* transcription of the cDNA or by direct transfection into permissive cells (Ruggli and Rice, 1999) (see Fig. 1). Transfection of transcribed genomic RNA, by direct *in vivo* inoculation of susceptible animals, has also been shown to rescue infectious virus (Gritsun and Gould, 1995) (see Fig. 2). In some cases, an antigenomic hepatitis delta virus ribozyme has been added at the 3′ end of the viral genome (Perrotta and Been, 1991). The ribozyme sequence is self-cleaved in cells and leaves the entire viral RNA genome intact and available for replication by the cellular machinery.

Whilst flavivirus infectious clones provide a powerful tool for studying many aspects of viruses, their construction as full-length cDNA molecules in bacterial vectors can be problematic, laborious and time consuming because they are often unstable, contain unwanted induced substitutions and may be toxic for bacteria due to viral protein expression. Various approaches have been employed to overcome these problems using different hosts, vectors, promoters or strategies to mutate cryptic bacterial promoter sites in the flavivirus genome.

3.1.1. Production of cDNA

Once the complete sequence of the genome is known, or alternatively the extreme 5′ and 3′ ends of the genome are known, obtaining a pool of high fidelity full-length cDNA is the first step in producing an infectious clone. Early methods usually used sub-cloning steps with the production of short cDNA fragments which were then linked together by ligation before being cloned as full-length cDNA molecules or in the case of the first flavivirus infectious clone, by the use of two plasmids and an *in vitro* ligation method to assemble full length templates for transcription (Lai et al., 1991; Rice et al., 1989). Subsequently, improvements in PCR enzyme fidelity and optimization of reaction conditions, led to major increases in the achievable length of authentic cDNA amplicons, finally enabling rapid amplification of genomic length cDNA (Barnes, 1994; Gritsun and Gould, 1995). As indicated

earlier, *de novo* synthesis of genomic length cDNA is now possible but currently is not the first method of choice. Regardless of which method is used, the sequence of the entire genome of the derived infectious clone must be determined and compared with the parent virus.

3.1.2. Choice of host for cloning the cDNA

3.1.2.1. Bacteria.

3.1.2.1.1. Different strains. The *E. coli* expression system is the most commonly used expression system for cloning of cDNA. The physiology and genetics of these bacteria are well-defined and a variety of different host strains with various genetic backgrounds are available giving them different specific characteristics to maximize the chance of successful cloning (Casali, 2003). Optimized strains may be selected because they contain one or more of several different antibiotic resistance genes (e.g., Chloramphenicol, Ampicillin, Tetracycline, Streptomycin), carry a chromosomal copy of a gene like the T7 RNA polymerase gene (BL21), can be specifically designed to clone large fragments of DNA (DH1, Topp-10), possess or do not possess specified restriction-modification systems or grow very fast (DH5α turbo). Some strains also harbor mutations that decrease the efficiency or invalid the recombination systems thus increasing the integrity of cloned DNA when direct or inverted repeats are present, have a high transformation efficiency (NovaBlue), do not methylate DNA (DM1) or even reduce the copy number of the plasmid for toxic proteins (ABLE C).

However, there is no perfect universal strain capable of amplifying with limited toxicity, any flavivirus infectious clone and it is therefore necessary to determine empirically which one is the most suitable for the sequence and the vector used. Thus different *E. coli* strains have been used to construct stable full-length DENV-4 cDNA (Lai et al., 1991) DENV1–4 chimeric virus (Bray and Lai, 1991), DENV-2 (Blaney et al., 2004a), DENV-3 (Blaney et al., 2004b), or TBEV (Pletnev et al., 1992). The strains used for the construction of the first major flavivirus infectious clones were previously described by Nicolas Ruggli and Charles Rice (Ruggli and Rice, 1999).

3.1.2.1.2. Different vectors. Since the beginning of molecular cloning in the early 1970s, the number of vectors created has increased enormously. Therefore, optimal vector choice is difficult but important to increase the success of obtaining the final construct. A variety of criteria should be taken into account such as the insert size, the copy number or the selectable marker.

Plasmid: The maintenance and propagation of plasmids into *E. coli* cells is an inefficient process because cells that do not contain a plasmid are at a growth advantage over those that do, given that they have to replicate both the chromosome and additional plasmid DNA. A method of selecting cells that have received a plasmid is therefore required and an appropriate selective pressure must be imposed for maintenance and enhancement of the plasmid. Almost all conventional plasmids use an antibiotic resistance gene, carried on the backbone of the vector, as a selectable marker. Thus, the addition of the appropriate antibiotic to the growth medium will kill the cells that do not contain the plasmid and produce a culture in which all cells do contain a plasmid. In many cases, the choice of antibiotic is not restricted. However, some cloning strains of *E. coli* are inherently resistant to some antibiotics. Thus, in these cases these antibiotics cannot be used to select cells carrying the plasmid of interest. The genotype of the desired cloning strain should be checked prior to cloning.

In theory, most general cloning plasmids can carry a DNA insert up to around 15 kb in size. Inserts in excess of this size may place constraints on efficient replication of the plasmids (particularly for high-copy-number vectors) and can cause problems with insert stability. Several types of vectors are available for cloning larger fragments of DNA.

Plasmids are maintained at different copy numbers, depending on the origin of replication. In the majority of cases in which a piece of DNA is cloned for conservation and amplification for subsequent manipulation, high yields of recombinant plasmid from *E. coli* cultures, are the preferred choice. However, a high-copy-number may cause problems for cloning DNA especially in the case of flaviviruses for which there exist, within their genomes, one or more cryptic prokaryotic promoters that facilitate the production of viral proteins which can be toxic to bacteria at high levels. Even if the protein is expressed poorly from the cloned DNA, the presence of many copies of the plasmid may raise the level of protein to toxic levels. In cases when recombinants are still obtained, their growth rate is often frustratingly slow, and the cloned foreign DNA sequences are often unstable (Blaney et al., 2006; Ruggli and Rice, 1999; Ward and Davidson, 2008). To solve these problems, low-copy-number vectors have been developed in which tightly regulated prokaryotic promoters are maintained at a low level of basal expression and prokaryotic transcription terminators prevent spurious transcription of foreign DNA sequences from upstream plasmid promoters.

This strategy has been used to obtain several infectious clones. For example with TBEV, the cDNA was cloned into the low copy number plasmid pBR322 (Gritsun and Gould, 1998) whereas the Murray Valley encephalitis virus (MVEV) prototype strain 1-51 was inserted into the low-copy-number plasmid vector pMC18 (Hurrelbrink et al., 1999) and the complete full-length cDNA of YFV was inserted under the control of an SP6 promoter in the low copy number plasmid pACYC177 (Bredenbeek et al., 2003). Similar approaches have been used for different strains of DENV-2 (Gualano et al., 1998; Kinney et al., 1997; Polo et al., 1997; Zhu et al., 2007), WNV (Shi et al., 2002; Yamshchikov et al., 2001b), KUNV (Khromykh and Westaway, 1994) or TBEV (Mandl et al., 1997). However, this does not always resolve the problem. For example, several attempts to construct genetically stable JEV cDNAs using low-, medium-, and high-copy-numbers of plasmids or special bacterial hosts all failed (Mishin et al., 2001; Sumiyoshi et al., 1992, 1995).

Bacterial artificial chromosome: Bacterial artificial chromosomes (BAC) are circular DNA molecules capable of maintaining very large DNA inserts up to 350 kb and can be used, instead of plasmids, as vectors for the assembly of infectious clones. They are based on the *E. coli* fertility factor (F-factor) replicon which is maintained as a circular supercoiled extra chromosomal single copy plasmid in the bacterial host (Monaco and Larin, 1994; O'Connor et al., 1989; Shizuya et al., 1992). Their high capacity suited their primary use for the construction of infectious clones of large DNA viruses such as baculoviruses (Luckow et al., 1993) or herpesvirus (Saeki et al., 1998).

However, BAC technology has also been extended to flaviviruses. Thus, full-length JEV cDNAs were successfully obtained by cloning the JEV genome into BAC vectors (Yun et al., 2003). The same method was also applied to YFV (van der Most et al., 1999), DENV-1 (Suzuki et al., 2007), and DENV-2 (Pierro et al., 2006; Usme-Ciro et al., 2014) suggesting that this method was most suited to bacteria that could resist the toxicity caused by flavivirus genomes.

Cosmid: BAC and plasmids are not the only vectors that can be used for infectious clone construction. Indeed, Zhang et al. (2001) used a cosmid vector, Expand Vector I (Zhang et al., 2001). These are conventional vectors that contain a small region of bacteriophage λ DNA containing the cohesive end site (cos). These vectors were designed to accommodate large inserts of 7–17 kb (Hohn and Collins, 1980). They have proved to be more suitable for cloning the full-length cDNA amplicon from JEV than other cloning vectors, such as pBR322, pKS, pUC or pGEM.

3.1.2.1.3. Choice of DNA-dependent RNA polymerase promoters. To efficiently generate viral RNAs from cDNA *in vitro*, *in cellulo* or *in vivo*, the viral sequences need to be flanked by a suitable promoter. The most commonly used promoters for flavivirus infectious clones are the bacteriophage promoters T7 and SP6, and to a lesser extent, the eukaryotic promoter of the human cytomegalovirus (referred here as CMV promoter) (see Fig. 1). Each has its own advantages. Bacteriophage promoters are used *in vitro* to produce large quantities of viral RNA from cDNA with bacteriophage RNA polymerases. The transcripts are then transfected directly into permissive cells or animals, and infectious virus can be recovered. In general, SP6 polymerase/promoters have lower efficiency than T7 *in vitro* transcription systems but under ideal conditions SP6 is roughly comparable with the others. However, these RNA polymerases are error prone, therefore, RNA transcripts cannot be viewed as genetically homogeneous populations (Boyer et al., 1992; Sooknanan et al., 1994).

The CMV promoter, on the other hand, enables viral RNA to be produced *in cellulo* after direct transfection by full-length cDNAs in eukaryotic cells. Such eukaryotic promoters have been successfully used for the production of "infectious DNA" of two strains of WNV (Khromykh et al., 2001; Pierson et al., 2005) as well as several positive strand RNA viruses, including polioviruses (Semler et al., 1984), foot-and-mouth disease virus (Beard et al., 1999), several alphaviruses (reviewed (Nougairede et al., 2013; Schlesinger and Dubensky, 1999)), coronaviruses (Almazan et al., 2000) and plant viruses (Boyer and Haenni, 1994; Johansen, 1996; Lopez-Moya and Garcia, 2000). While the use of this promoter confers some logistical advantages over bacteriophage promoters that require the production of RNA *in vitro*, the problem of the stability and the toxicity in bacteria still remain and are made particularly acute in *E. coli* by the transcriptional activity of eukaryotic promoters (Antonucci et al., 1989; Davis and Huang, 1988) while T7 and SP6 promoters are not transcriptionally active in *E. coli*.

3.1.2.1.4. General problems involved in deriving infectious clones. Construction of infectious clones enables direct genetic manipulation of viral genomes and is a valuable tool of modern experimental virology. However, its use and practical importance are often limited due to instability of the construct during propagation in *E. coli*. This problem has been particularly evident when developing flavivirus infectious clones. As a consequence, some virus infectious clones are either almost impossible to construct, or are very difficult to maintain in *E. coli* due to their predisposition to spontaneous rearrangements and/or to acquisition of stabilizing mutations. The most plausible explanation is that such instability results from unanticipated expression of viral cDNA encoding products toxic to the bacterial host. Thus, different strategies have been elaborated based on the observation that artificially introduced deletions, insertions or frameshifts can alleviate the cloning problems, either by increasing the stability of the construct, decreasing the transcriptional activity of the promoter or the cryptic expression of viral proteins.

Reduction of toxicity in bacteria: To circumvent the presence of promoter-like sequences inside the viral genome or foreign gene cassettes, advantage was taken of the major difference in mRNA formation between prokaryotes and eukaryotes by using eukaryotic RNA processing mechanisms as the essential part of the clone stabilization strategy (Yamshchikov et al., 2001a). This approach was conceived from the observation that a single nonsense mutation, that result in the apparition of a premature stop codon, was often sufficient to stabilize the entire construct presumably by preventing synthesis of toxic products. Hence, temporary ablation of such deleterious expression at the translational level by interrupting the ORF, with the help of an intron containing stop codon(s), was beneficial during propagation of recombinant plasmids in *E. coli*. The intron effectively blocked synthesis of toxic products in the bacterial host, even if spurious transcription resulted in formation of mRNA. Nuclear transcription in eukaryotic cells, accompanied by splicing of the primary transcripts, would lead to precise excision of the intron and restoration of the original ORF. For virus infectious clones and viral replicon-based expression vectors, subsequent export of mRNA to the cytoplasm and its translation resulted in synthesis of viral proteins, which initiated the viral infectious cycle or replication of vector RNA. Using this approach, deleterious effects of spurious transcription from eukaryotic promoters in bacterial cells were eliminated at the translational level by insertion of an intron(s) into viral cDNA cassettes. Nevertheless, in some cases modification of the viral cassette was not always feasible possibly because the problem for implementation of the proposed approach was associated with the potential existence of cryptic splice sites in viral RNA genomes (Shiu et al., 1997). However, when potential sites were found this did not appear to have a substantial impact on the functional properties of the designed infectious DNA constructs.

Interruption of a viral open reading frame either by frameshift mutations or insertion of introns leads to substantial stabilization of plasmids containing viral genome cassettes under the control of eukaryotic promoters (Johansen, 1996; Lopez-Moya and Garcia, 2000; Olsen and Johansen, 2001). This suggests that spurious transcription from eukaryotic promoters in *E. coli* (Antonucci et al., 1989; Davis and Huang, 1988) may substantially contribute to the observed instability reported.

In another approach, the investigators tried to determine if a decrease of spurious transcription could eliminate the destabilizing effect of eukaryotic promoters on "infectious DNA" constructs. They thus described an alternative "minimal" approach based on the manipulation of the promoter to minimize its activity in bacteria and reduce its deleterious effects (Mishin et al., 2001).

The CMV promoter, often used to drive expression of engineered constructs in a variety of mammalian cells (Foecking and Hofstetter, 1986) consists of a transcriptionally active minimal promoter region immediately adjacent to the RNA transcription initiation codon, and an upstream enhancer element, which only displays transcriptional activity in mammalian cells (Thomsen et al., 1984). Using the knowledge that certain regulatory elements of eukaryotic genes, such as enhancers, can influence gene expression in position- and orientation-independent manner (Jonsson et al., 1994) the enhancer region was separated from the promoter region and moved either directly upstream of the promoter or immediately following the polyadenylation site. The deletion of the enhancer led to a 5- to 6-fold reduction of gene expression. In contrast, the relocalization of the CMV enhancer downstream of the reporter gene had no discernible effect on gene production in *E. coli*. Insertion of the CMV enhancer after the polyadenylation signal resulted in a 10- to 100-fold increase of the transcription rate in mammalian cells without affecting the stability of constructs in *E. coli*.

While this approach cannot resolve instability problems associated with the presence in viral genome of cDNA prokaryotic promoter-like elements (Johansen, 1996; Lopez-Moya and Garcia, 2000; Olsen and Johansen, 2001), it provides, together with the intron-based stabilization approach, flexibility and a choice in the design strategy.

Finally, Pu et al. (2011) developed a method for constructing stable full-length flavivirus cDNA by reducing the intrinsic toxicity of the viral genome sequence in *E. coli* (Pu et al., 2011). They demonstrated that the instability of the DENV-2 and JEV cDNA clones in bacteria is due to the toxicity of the cryptic expression of viral proteins by multiple putative *E. coli* promoter (ECP) sequences that are embedded in the viral genome. Hence, they designed a new approach to assembling infectious DENV-2 and JEV cDNA clones by introducing silent mutations into the ECP sequences within

the DENV-2 and JEV genomes. These silent mutations reduced ECP activity, which resulted in the stabilization of the full-length DENV-2 and JEV cDNA clones in *E. coli*.

Recently, a new approach to propagate DENV-2 and JEV infectious cDNA clones in bacteria was described (Pu et al., 2014). Tandem repeat sequences (seven repeated tetracycline-response element (7XTRE)) were introduced upstream of a minimal CMV promoter sequence (Cai et al., 2005) followed by the viral genome. The cryptic expression of DENV-2 or JEV proteins in bacteria was greatly reduced which increased stability to the cDNA clones without altering the sequence of the viral genome.

Yeast: Some full-length flaviviral cDNA genomes, unstable in *E. coli,* were successfully cloned in eukaryotic systems which are often more tolerant than bacteria to toxic flavivirus sequences presumably because they promote less cryptic viral protein expression. The yeast *Saccharomyces cerevisiae* is a popular eukaryotic host in cases for which viral cDNA was assembled by homologous recombination. The two common strategies for cloning DNA in yeast are the assembly of yeast artificial chromosomes (YACs) and the use of yeast-*E. coli* shuttle vectors such as pRS424. Using this approach, Polo et al. (1997) assembled full-length DENV-2 cDNA in yeast by recombination *in vivo*, followed by transformation of *E. coli* to produce plasmid DNA for *in vitro* transcription (Polo et al., 1997). Similar approach have also been applied for DENV-3 (Santos et al., 2013). Even if the problems with deleterious effects and instability in the *E. coli* host were not completely eliminated, this method enabled recovery of infectious full-length RNA transcripts. Of the two methods used to assemble the full-length DENV clone, the shuttle vector approach was simpler and more reliable than the YAC approach essentially due to the need of a supplementary cloning step before the infectious clone could be recovered from the YAC in bacteria. The same method has also been used to assemble full-length DENV-1 (Puri et al., 2000) and DENV-4 (Kelly et al., 2010) cDNAs.

3.2. Bacteria-free approaches

Current methodologies for construction of infectious cDNA clones are time-consuming, unpredictable and laborious processes frequently associated with undesirable mutations or unstable/toxic clones in bacteria. In order to avoid the need of using a bacterial host and thus to overcome the problems related to their use, bacteria-free approaches have been developed.

3.2.1. Long PCR

Genetically engineered infectious viral RNA can be obtained by direct transcription using a full-length cDNA template from either a long RT-PCR or fusion of overlapping RT-PCR fragments without cloning (see Fig. 2). Using this rapid approach, the problematic process of construction of a full-length cDNA clone by repeated ligation can be avoided and recombinant viruses can be made within days.

This strategy was first used by Gritsun and Gould (1995) for the Siberian subtype Vs of the TBEV (Gritsun and Gould, 1995). The procedure developed was based on the high fidelity, high yield, long PCR protocols described by Barnes (1994) and Cheng et al. (1994) and utilized a mixture of two thermostable DNA polymerases, one of which possessed a 3′–5′ exonuclease "proofreading" activity, to produce two overlapping cDNA products (5.7 and 5.2 kb in length) representing the full-length genome of the virus (Barnes, 1994; Cheng et al., 1994). The 5′ half of the cDNA was designed to contain an SP6 promoter added in the first forward primer. Full-length cDNA of Vs virus was then produced either by ligation of the two overlapping DNA molecules in a unique restriction site or by fusion PCR. When directly inoculated intracerebrally into mice, the *in vitro* transcribed RNA from these templates was infectious. This method avoided the need for virus purification and cDNA cloning procedures and shortened the time to produce engineered virus from years to days. This was the first report in which an infectious RNA virus has been derived by PCR without intermediate cloning of the cDNA in microbial vectors.

3.2.2. Circular polymerase extension reaction and Gibson assembly

As an alternative, Edmonds et al. (2013) developed another approach for rapid generation of KUNV infectious cDNAs that completely eliminates the need of plasmid DNA propagation in bacteria and *in vitro* RNA transcription (Edmonds et al., 2013) (see Fig. 3).

This approach is based on using a circular polymerase extension cloning (CPEC) reaction with Phusion high-fidelity DNA polymerase to assemble multiple RT-PCR amplicons in the right order in a vector. For that purpose, the RT-PCR fragments contain overlapping ends and the first and the last RT-PCR amplicons also have overlapping ends with the vector fragment to ensure that when the fragments and the vector are mixed and denatured, the overlapping ends anneal to each other. DNA polymerase then extends the fragments, using the neighboring fragment as a primer. If the overlapping fragments are designed to have similar annealing temperatures, this reaction generates a circular end product. The generated circular product can then be transfected directly into competent cells without any additional manipulations since the *in vitro* RNA transcription step was eliminated by replacing SP6 or T7 polymerase promoters with the CMV promoter, thus allowing transcription of viral RNA in cells directly from plasmid DNAs by the cellular RNA polymerase II (Edmonds et al., 2013).

Based on the same principle, Siridechadilok et al. (2013) used the Gibson assembly, a combination of three enzymes activity (exonuclease, DNA polymerase and DNA ligase) to assemble multiple overlapping DNA fragments in an isothermal and single-reaction, to reconstruct the DENV genome from multiple PCR products, amplified from cDNA, onto an expression plasmid with a CMV promoter that precisely initiated transcription on the virus genome sequence and a terminator, such as hepatitis D virus (HDV) ribozyme that accurately generated 3′ end of the transcribed viral RNA (Siridechadilok et al., 2013) (see Fig. 4).

3.2.3. Infectious subgenomic amplicons

Recently, Aubry et al. (2014) described a new reverse genetics method designated ISA (Infectious-Subgenomic-Amplicons) that facilitates the production of infectious wild-type or genetically modified animal RNA viruses from genomic DNA material, in a variety of forms, including pre-existing infectious clones, viral RNA or *de novo* synthesized DNA genomic sequences (Aubry et al., 2014) (see Fig. 5). This procedure does not require cloning, propagation of cDNA into bacteria or *in vitro* RNA transcription. Entire genomes have been amplified by PCR in 3 DNA fragments of approximately 4 kb, each with 70–100 bp overlapping regions. The first and last fragments were flanked respectively in 5′ and 3′ by the CMV promoter and the hepatitis delta ribozyme followed by the simian virus 40 polyadenylation signal. PCR products were mixed and transfected into permissive cells which enabled the recovery of infectious viruses. Unlike other bacterium free approaches, the ISA method does not require any additional step beside the PCR amplification needed to obtain the different cDNA fragments. Instead, the process proceeds naturally by *in cellulo* recombination, which greatly facilitates and shortens the procedure.

In the initial study, the method was successfully used for WNV, YFV, JEV, TBEV, DENV, Chikungunya virus (*Alphavirus*) and Coxsackievirus B3 (*Picornavirus*).

4. Impacts/utilisations

4.1. Reverse genetics methods and clonality of viral populations

Viral RNA polymerases characteristically exhibit low fidelity which results in relatively high mutation rates during viral RNA replication. Consequently, newly generated populations of most RNA viruses are extremely heterogeneous. This intra-population variability has been described as a "mutant spectrum". While the phenotype of a given virus has been attributed to the consensus sequence, it is now established that the mutant spectrum as a whole, is responsible for the phenotype (Ciota et al., 2007; Holmes and Moya, 2002). In the case of arboviruses, an additional factor is involved. These viral agents alternate between arthropod vectors and vertebrate hosts. Consequently, the mutant spectrum is likely to reflect these selective constraints, resulting in variant populations that ensure selection of phenotypes efficient in replication and transmission between both hosts (Ciota et al., 2008, 2007; Coffey and Vignuzzi, 2011; Coffey et al., 2008).

Most current reverse genetic systems for engineering viruses use cloning methods during the experimental procedure. The viral cDNA is cloned into a vector (plasmid, BAC, cosmid), and infectious viruses are then recovered either by *in vitro* transcription using bacteriophage RNA polymerase and transfection into permissive cells, or they are directly recovered from transfected cells through the use of a eukaryotic transcriptional promoter. The transfected cDNA used to recover infectious viruses can be considered as clonal and consequently, at this stage, the mutant spectrum present in the original virus population no longer exists. Currently, viral populations approximating to clonality can be obtained using either of the following two methods, production of viral genomic cDNA cloned into a plasmid under the control of a eukaryote promoter or clonal subgenomic cDNA fragments (e.g., plasmid fragments used instead of PCR products using the ISA method). Potential impacts arising from the depletion of mutant spectra could result in modification of the viral phenotype. For example, it was observed that viruses recovered using reverse genetics initiated from clonal populations, replicated more slowly than their corresponding parent non-cloned ancestors (Coffey and Vignuzzi, 2011; Kinney et al., 1997; Pierro et al., 2006) or they exhibited reduced neurovirulence for rodents (Hayasaka et al., 2004). However, based on consensus sequencing, mutations could not be identified to account for these different phenotypes. These examples illustrate some of the potential limitations of the reverse genetics and sequencing methods used to investigate the basis of virus pathogenesis.

In contrast, the new methods described above which do not involve bacterial cloning of the viral cDNA, provide the opportunity to conserve the mutant spectrum present in the original viral sample (e.g., a clinical sample or cell culture isolate) (Aubry et al., 2014; Edmonds et al., 2013; Siridechadilok et al., 2013).Alternatively, other methods can potentially generate genetic variability completely different from the initial diversity of the source material. For example, error-prone DNA-dependent RNA polymerases such as T7 or SP6 introduce random mutations during *in vitro* transcription of viral RNA genomes from cloned cDNA. Moreover, DNA dependent DNA polymerases can also generate errors. Thus, the possible use of PCR enzymes that exhibit different levels of fidelity could be considered as a means of regulating the variant composition within the mutant spectrum and presumably, changing the phenotype of the recovered virus. Indeed, one could visualize the use of such methods to investigate the underlying basis of viral pathogenesis and in the case of arboviruses to examine the impact of altered mutant spectra on virus–vector–host interactions.

Keeping this in mind and at the same time wishing to exploit the potential versatility of reverse genetics methods, one can imagine how different questions need to be raised before the elaboration of any experiment. That will determine the choice of the most suitable reverse genetic system, each one of them possessing its own advantages and drawbacks. For example, if the objective is to study the effect of one mutation, working with a clonal viral population could be appropriate and it would be advantageous to work with infectious clones or an equivalent method. However, if the objective is to study the interactions between host and virus, then it would be interesting to use reverse genetics methods that conserve the original mutant spectrum to mimic more closely what is happening during the natural cycle of the virus.

4.2. Reverse genetics methods as research tools

Reverse genetics systems allow artificial manipulation of viral genomes by site-directed mutagenesis, deletion/insertion, and rearrangement. Studying the phenotype of the viruses generated using these methods has allowed the scientific community to gain valuable knowledge related to the replication, biological characteristics, and pathogeneses of these viral agents. Here, we describe recent selected advances arising from the study of flaviviruses based on the application of reverse genetics (Table 1) (earlier findings were described by Ruggli and Rice (Ruggli and Rice, 1999)).

4.2.1. Studying point mutations

Reverse genetics enables high fidelity production of infectious viruses from cloned viral cDNA, by utilizing the very low mutation rate of viral cDNA-bearing plasmids during their amplification in bacteria. This provides the possibility to investigate the consequences on replicative fitness of the introduction of a single mutation. Accordingly, a large number of genetically defined viral mutants have been constructed to study a variety of flaviviruses, including YFV (Muylaert et al., 1997), DENV (Leardkamolkarn et al., 2010; Matusan et al., 2001), JEV (Arroyo et al., 2001; Sumiyoshi et al., 1995; Ye et al., 2012; Zhao et al., 2005), WNV (Arroyo et al., 2004; Martin-Acebes et al., 2013; Van Slyke et al., 2012) or TBEV (Gritsun et al., 2001; Mandl et al., 2000; Yoshii et al., 2004).

As examples, point mutations engineered into the prM or E proteins of DENV-2/WNV chimeric viruses have been shown to improve viability and viral yields in cell cultures (Huang et al., 2005). DENV-2 viruses containing single point substitution in the 5′ UTR exhibited reduced replicative fitness *in cellulo* and reduced neurovirulence for newborn mice (Leardkamolkarn et al., 2010). This observation was confirmed for several other flaviviruses when deletions were engineered into the 3′ and 5′ UTR causing attenuation in cell culture and in animals (Blaney et al., 2010, 2008; Cahour et al., 1995; Mandl et al., 1998; Men et al., 1996; Zeng et al., 1998). Van Slyke et al. (2012) engineered point mutations in the WNV NS5 gene which encodes the RNA polymerase (Van Slyke et al., 2012). The point mutations were positioned remote from the polymerase active site. This resulted in reduced replicative fitness *in cellulo* and attenuation of neurovirulence *in vivo*. Five shared point mutations were introduced in the hinge region spanning domains I and II of the E glycoprotein of JEV (residues E107, E138, E279, E315, E439) and WNV (residues E107, E138, and the corresponding E280, E316 and E440) (Arroyo et al., 2001, 2004) and also 3 mutations were introduced in the upperlateral surface of domain III of TBEV (residues E308, E310, E311) (Mandl et al., 2000). In each of these examples the viruses showed reduced neurovirulence in mice. However, the single mutation located in the hinge region, E279, of JEV increased neurovirulence (Monath et al., 2002). Thus, each study identified a direct association between a specific viral genomic region or specific site and the corresponding biological properties of the virus.

Table 1
List of the different studies quoted in Section 4 .

Impact/utilisations	Virus	References
Single mutations	YFV	Muylaert et al. (1997)
	DENV	Blaney et al. (2008, 2010), Cahour et al. (1995), de Wispelaere and Yang (2012), Huang et al. (2005), Hung et al. (2004), Leardkamolkarn et al. (2010), Matusan et al. (2001), Men et al. (1996), Modis et al. (2004) and Zeng et al. (1998);
	JEV	Arroyo et al. (2001), Liang et al. (2009), Lin et al. (2006), Liu et al. (2004), Monath et al. (2002), Sumiyoshi et al. (1995) and Ye et al. (2012)
	WNV	Arroyo et al. (2004), Huang et al. (2005), Martin-Acebes et al. (2013) and Van Slyke et al. (2012)
	TBEV	Gritsun et al. (2001), Mandl et al. (1998, 2000) and Yoshii et al. (2004)
	MVEV	Hurrelbrink and McMinn (2001)
Virulence determinants	DENV	Bray et al. (1998), Grant et al. (2011), Gualano et al. (1998), Hanley et al. (2002), Leardkamolkarn et al. (2010) and Prestwood et al. (2008)
	JEV	Tajima et al. (2010) and Yamaguchi et al. (2011)
	WNV	Audsley et al. (2011), Borisevich et al. (2006), Pijlman et al. (2008), Shirato et al. (2004), Whiteman et al. (2011), Wicker et al. (2006, 2012), Yu et al. (2008) and Zhang et al. (2006)
	TBEV	Mandl (2005)
	LGTV	Rumyantsev et al. (2006)
	KUNV	Audsley et al. (2011) and Liu et al. (2006)
Virus/host cell machinery interactions	DENV	Duan et al. (2008)
	WNV	Davis et al. (2007) and Emara et al. (2008)
Mechanisms of viral immune evasion	WNV	Liu et al. (2006) and Schuessler et al. (2012)
Host specificity	YFV	Charlier et al. (2010)
	DENV	Charlier et al. (2010) and Tumban et al. (2011)
	WNV	Saiyasombat et al. (2014)
	LGTV	Tumban et al. (2011) and Saiyasombat et al. (2014)
	MODV	Charlier et al. (2010) and Saiyasombat et al. (2014)
Reporter genes	DENV	Schoggins et al. (2012) and Zou et al. (2011)
	WNV	Pierson et al. (2005) and Puig-Basagoiti et al. (2005)
Viral proteins	DENV	Crabtree et al. (2005), Erb et al. (2010), Pryor and Wright (1993, 1994) and Pryor et al. (1998)
	WNV	Beasley et al. (2005)
	TBEV	Kofler et al. (2003)
	KUNV	Hall et al. (1999) and Liu et al. (2003)
	MVEV	Clark et al. (2007)
Cyclization sequences and RNA secondary structures	YFV	Bredenbeek et al. (2003)
	DENV	Alvarez et al. (2008), Liu et al. (2013) and Men et al. (1996)
	TBEV	Mandl et al. (1998) and Tuplin et al. (2011)
	KUNV	Khromykh and Westaway (1997)
Chimeric vaccines	YFV	Arroyo et al. (2004), Caufour et al. (2001), Chambers et al. (1999), Guirakhoo et al. (2000, 2001, 2006), Monath et al. (1999, 2001, 2003, 2006), Pugachev et al. (2004) and Van Der Most et al. (2000);
	DENV	Bray and Lai (1991), Bray et al. (1996, 1998), Caufour et al. (2001), Chen et al. (1995), Guirakhoo et al. (2000, 2001, 2006), Li et al. (2013a), Maximova et al. (2014), Pletnev et al. (2002) and Van Der Most et al. (2000)
	JEV	Chambers et al. (1999), Li et al. (2013a,b) and Monath et al. (1999, 2003)
	WNV	Arroyo et al. (2004), Li et al. (2013b), Maximova et al. (2014), Monath et al. (2001, 2006), Pletnev et al. (2002) and Wang et al. (2014)
	TBEV	Wang et al. (2014)
	SLEV	Pugachev et al. (2004)
Antivirals	DENV	Qing et al. (2010) and Yang et al. (2014)

In addition to linking specific mutations to alteration of viral replication or virulence characteristics, reverse genetics has also enabled precise identification of the specific stage within the viral replication cycle that is altered. In a remarkable example of this, a silent mutation in the NS2A gene of the vaccine strain of JEV (JEV-SA-14-14-2) has been shown to abolish the formation of the large NS1′ protein by destabilizing a conserved RNA pseudoknot secondary structure responsible for the occurrence of a ribosomal frameshift, thus reducing neurovirulence and neuroinvasiveness in mice (Ye et al., 2012). In another study, mutation E138K in JEV domain I of the E protein, had a direct impact on viral spread from cell to cell by increasing virus-cell interactions and decreasing viral entry efficiency (Zhao et al., 2005) resulting in reduced expression kinetics of viral proteins, including the NS5 protein, an Interferon (IFN) antagonist (Lin et al., 2006), which in turn, leads to reduced capacity to block IFN signaling and increases the attenuation phenotype (Liang et al., 2009). Other studies on JEV, MVEV and DENV have indicated that mutations in domains II and III of the E protein can impair virus binding and entry into host cells (Hung et al., 2004; Hurrelbrink and McMinn, 2001; Liu et al., 2004; Modis et al., 2004). More recently, De Wispelaere and Yang (2012) showed that mutation of three conserved residues in the Domain I/Domain III linker of the DENV-2 E protein greatly impaired viral production by abolishing formation of viral particles within the cells (de Wispelaere and Yang, 2012).

4.2.2. Host/pathogen interactions

The ease with which infectious clones can now be genetically modified has provided the opportunity to gain new insights into the nature of virus–host interactions in the context of virulence determinants, virus–host cell interactions and the mechanisms of evasion that underly host specificity.

4.2.2.1. Virulence determinants. By introducing mutations or constructing intratypic/heterotypic chimeric viruses, numerous virulence determinants have been identified and analyzed to understand the mechanistic basis behind these altered phenotypes (reviews (Hurrelbrink and McMinn, 2003; Mandl, 2005)).

Using an infectious clone of WNV, Pijlman et al. (2008) showed that all members of the genus *Flavivirus* produce a unique, abundant, non-coding subgenomic RNA element (sfRNA), derived from the 5′-end of the 3′ UTR that protects downstream RNA from degradation by cellular ribonucleases (Pijlman et al., 2008). This sfRNA has been shown to play an important role in the virus replication cycle and contributes to viral cyto- and host-pathogenicity. Other WNV genetically engineered viral mutants have been generated to enable the identification of a large number of virulence determinants within the structural/non-structural genes and the 3′ UTR of the viral RNA genome (Audsley et al., 2011; Borisevich et al., 2006; Liu et al., 2006; Shirato et al., 2004; Whiteman et al., 2011; Wicker et al., 2006, 2012; Yu et al., 2008; Zhang et al., 2006).

Grant et al. (2011) showed that a single amino-acid substitution NS4B-F52L in the mouse-virulent DENV-2 strain D2Y98P-PP1 significantly reduced the efficacy of viral RNA synthesis in mammalian cells and completely abolished the virulence/pathogenesis in an AG129 mouse model (Grant et al., 2011). They also showed that the reverse substitution in a non-virulent DENV-2 strain TSV01 was sufficient to make this strain virulent in the same mouse model. Several other viral determinants modulating the intrinsic virulence of DENV, for mice, have been identified and shown to affect either the replication efficiency (Leardkamolkarn et al., 2010) or the binding properties (Bray et al., 1998; Gualano et al., 1998; Prestwood et al., 2008). Similar results were obtained with the JEV strain Mie/41/2002 in which the E-S123R amino-acid substitution exhibited increased virulence for mice (Tajima et al., 2010). Notably, a correlation between growth properties *in cellulo* and virulence *in vivo* was not always observed. Indeed, Yamaguchi et al. (2011) revealed that an amino acid substitution NS4A-V3I of the JEV strain Mie/40/2004 increased its virulence in mice without influencing the growth rate in cell culture (Yamaguchi et al., 2011).

Molecular determinants of neuroinvasiveness and neurovirulence have also been studied. Rumyantsev et al. (2006) demonstrated inhibition of neuroinvasiveness with Langat virus (LGTV) strain TP21 and identified 4 amino acid substitutions located either in the E protein (S267L; K315E; N389D) or the NS3 protein (K46E) that are independently responsible for the abolition of neuroinvasiveness (Rumyantsev et al., 2006). Likewise, Hanley et al. (demonstrated that replacement of several paired charged amino acids, by alanine, in the NS5 polymerase protein decreased DENV-4 neurovirulence in suckling mice (Hanley et al., 2002)). Moreover, a large number of specific mutations in the E protein domains of TBEV, JEV, MVEV or YFV have been shown to affect either neuroinvasiveness or neurovirulence in mice (reviews (Hurrelbrink and McMinn, 2003; Mandl, 2005)).

4.2.2.2. Virus/host cell machinery interactions. By the introduction of various amino acid substitutions into the Elongation factor 1-alpha (eEF1A) binding site or into adjacent areas of the 3′-terminal Stem–Loop (3′ SL) in the 3′ UTR of a WNV infectious clone, it was shown that mutations which decreased the binding efficiency of eEF1A to the viral 3′ SL *in vitro* had a negative effect on viral minus-strand RNA synthesis *in cellulo* and *vice versa* for mutations that increased binding efficiency of eEF1A (Davis et al., 2007). They also showed that eEF1A co-localized with viral replication complexes in infected cells which strongly suggest that interaction between the cellular protein eEF1a and the 3′ SL of the WNV genomic RNA facilitates viral minus-strand RNA synthesis.

Other interactions between the 3′ SL and host cellular factor have been reported and their roles identified. Using an approach similar to that described above, the interaction between the T-cell intracellular antigen-related (TIAR) protein and T-cell intracellular antigen-1 (TIA-1) protein with the WNV minus 3′ SL RNA (3′ (−)SL RNA) was characterized (Emara et al., 2008). Using *in vitro* experiments the specific binding sites on the WNV 3′ (−)SL RNA were identified and the effects of mutations on virus replication within these binding sites were analyzed. The efficiency of genomic RNA synthesis and virus production progressively decreased with decreasing *in vitro* binding efficiency of TIAR/TIA-1 for mutant 3′ (−)SL RNAs. In these experiments neither TIA-1 nor TIAR was found to bind to the 3′ (+)SL or 5′ (+)SL of the genomic RNA. The fact that several mutant RNAs that inefficiently interacted with TIAR/TIA-1 *in vitro* rapidly reverted *in vivo*, indicated that they could replicate at a low level. This suggests that interaction between TIAR/TIA-1 and the viral 3′ (−)SL RNA is not required for initial low-level symmetric RNA replication but instead facilitates the subsequent asymmetric amplification of genome RNA from the minus-strand template.

In another study, the domains on prM protein that might be responsible for its interaction with the vacuolar ATPase (V-ATPase) were analyzed. Using several DENV with deletions in the prM region, critical residues located in the pr portion of this protein which are highly conserved among the members of flavivirus and responsible for the binding of the V-ATPase, were identified (Duan et al., 2008). The use of a specific inhibitor of V-ATPase that inhibits endosome and lysosome acidification demonstrated that this interaction plays an important role in mediating low-pH dependent entry of DENV and also in establishing a suitable pH environment that will facilitate efficient virus secretion.

4.2.2.3. Mechanisms of viral immune evasion. Research that focuses on identification and understanding of the mechanisms involved in viral evasion of host immune responses has also benefited significantly through the development of improved reverse genetics methodologies. Thus, it was demonstrated that incorporation of an NS2A-A30P mutation into the KUNV genome results in a mutant virus that elicits more rapid induction and higher levels of synthesis of IFN-α/β in infected human A549 cells when compared with unmodified KUNV. This introduced mutation induces drastic diminution of KUNV neuroinvasiveness and neurovirulence in mice and this virus was also partially attenuated in IFN-$\alpha/\beta\gamma$ receptor knockout mice. Thus, the A30P mutation may also play a role in more efficient activation of other antiviral pathways (Liu et al., 2006).

It has also been shown that a mutant KUNV deficient in the production of sfRNA replicated poorly in WT mice and in Mouse Embryonic Fibroblasts (MEFs). However, the virulence of the mutant virus was largely rescued in mice with a combined deficiency in major factors involved in the type I IFN response, the transcriptional regulators IRF-3 and IRF-7, which act downstream of IPS-1 and MyD88, or a deficiency of the type I alpha/beta interferon receptor (IFNAR) suggesting a contribution for sfRNA in overcoming the antiviral response mediated by type I IFN (Schuessler et al., 2012). Also, remaining differences in virulence between WT and sfRNA-deficient virus in IFN response-defective mice suggest limited contribution of an IFN-independent antiviral response in restricting virulence of sfRNA-deficient virus.

4.2.2.4. Host specificity. Many of the recognized flaviviruses are arboviruses, i.e., they are transmitted to vertebrates by the bite of an infected arthropod. However, others, including insect-specific flaviviruses (ISFV) and non-known-vector (NKV) flaviviruses are not arboviruses. The viral determinants responsible for host tropism and vector specificity remain largely undefined. In order to explore the role of the UTRs in host specificity, chimeric genomes were generated in which the 5′ UTR, capsid and/or 3′ UTR encoding regions of mosquito-borne DENV-4 were replaced, separately or in combination, with those of tick-borne LGTV. None of the chimeric genomes yielded detectable virus following transfection. Replacement of the variable region (VR) in the DENV-4 3′ UTR (DENV-4-LGTswapVR) with that of LGTV showed lower replication

than its wild-type parents in mammalian cells but not in mosquito cells and was able to infect mosquitoes *in vivo*. Moreover, LGTV was able to infect ticks while DENV-4 or DENV-4-LGTswapVR did not (Tumban et al., 2011). These results suggest that UTRs cannot be exchanged between tick-borne and mosquito-borne flaviviruses and that the 3′ VR did not affect mode of transmission.

To study whether or not the viral E protein plays a role in the (in)ability of flaviviruses to infect mosquito cells, the replication of two chimeric flaviviruses was monitored. For these experiments, the prM+E region or the AnchC+prM+E region of the mosquito-borne flaviviruses YFV-17D or the DENV-2 were replaced by the corresponding region of the NKV flavivirus Modoc virus (MODV). These chimeric viruses replicated efficiently in mammalian (Vero-B) and mosquito (C6/36) cells, whereas infectious MODV RNA failed to replicate in mosquito cells (Charlier et al., 2010). These observations suggest that the inability of NKV flaviviruses to replicate in arthropod cells is not determined by the viral E proteins but appears to occur at a post-entry stage most likely due to the differences in host protein profiles between mammalian and arthropods cells. The results obtained by Saiyasombat et al. (2014) using a similar approach corroborate these observations and indicate that the vertebrate host specificity of MODV could be conditioned by genetic elements located outside of the prM+E region (Saiyasombat et al., 2014).

4.2.2.5. Reporter genes. The ability to monitor rapidly and quantitatively, virus replication by virtue of reporter gene activity provides numerous applications for the analysis of different facets of flavivirus biology, both *in vitro* and *in vivo*. Indeed, a detection system, such as a recombinant virus expressing a reporter gene that can be monitored in real-time may shorten the amount of time needed to conduct antiviral assays for the development of novel therapeutics. Another powerful application is the study of virus tropism *in vivo*. The reporter gene can be used to identify the initial cellular targets during an infection, how these cells migrate after virus exposure or the mechanism by which a virus can penetrate the central nervous system.

Infectious molecular clones capable of expressing high levels of a reporter gene in infected cells have also been described. Thus, the gene encoding green fluorescent protein (GFP) was cloned into the 3′ UTR of the viral genome under the translational control of the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV). Transfection of cells with this infectious DNA plasmid resulted in the production of infectious WNV capable of mediating high-level expression of GFP in several different cell types (Pierson et al., 2005). This approach has also been used to clone a *Renilla* luciferase reporter gene (Puig-Basagoiti et al., 2005).

Zou et al. (2011) on the other hand, used a different approach to construct a cDNA clone of DENV-2 that encodes the *Renilla* luciferase reporter gene (Zou et al., 2011). Indeed, a fragment encompassing the first 38 amino acids of the capsid protein, a *Renilla* luciferase gene, and a 2A sequence from the foot-and-mouth disease virus (FMDV2A) was inserted at the junction between the 5′ UTR and the ORF of the viral genome. The duplication of the N-terminal 38 amino acids of the capsid protein was used to maintain RNA elements that are required for genome cyclization (Alvarez et al., 2008, 2005a; Friebe and Harris, 2010) while the FMDV2A sequence was used to ensure that the luciferase protein was properly processed.

A similar approach was used by Schoggins et al. (2012) for the generation of infectious DENV-2 that express either GFP or firefly luciferase. These constructs were then used to follow the dynamics of DENV infection in mice by bioluminescence imaging and identify IFN-stimulated genes (ISGs) with anti-DENV activity by screening the DENV-GFP against a library of more than 350 ISGs (Schoggins et al., 2012).

However, it is worth noting that although these constructs present great potential, they often remained unstable through continuous cell passaging and/or exhibited decreased fitness compared to wild-type.

4.2.3. Viral proteins

4.2.3.1. Functions of NS2A and NS3 proteins. Two amino acid substitutions, NS2A-I59N and NS3-Y518H, that contributed to severely impaired generation of virus infectivity were identified in reverse genetics studies that utilized the infectious clone of KUNV (Liu et al., 2003). Using site-directed mutagenesis, it was demonstrated that the mutation located in the NS3 protein induced severe inhibition of RNA replication while the introduced mutation in the NS2A protein blocked virus assembly and/or secretion. In addition, sequence analysis of the recovered viruses showed that both amino acids had reverted to the wild-type sequence demonstrating the functional importance of these residues in viral replication and/or viral assembly.

4.2.3.2. Importance of the capsid protein internal hydrophobic domain. The isolation of viable mutants of TBEV carrying extending deletions in the capsid protein demonstrated that infectious particles can be formed even if the entire central hydrophobic domain is removed. However, these results showed that large deletions were tolerated only following the acquisition of additional mutations that increased the hydrophobicity of the protein suggesting that they may compensate for the loss of the central hydrophobic domain (Kofler et al., 2003). These results corroborate the notion that hydrophobic interactions of protein C are essential for the assembly of infectious particles but illustrate that individual residues within the central hydrophobic domain are not absolutely required for infectivity.

4.2.3.3. Importance of the domain-III FG loop for infection of mammalian and mosquito cells. An infectious clone of DENV-2 was used to investigate the importance of the extended loop motif between the F and G beta strands (FG loop) located in domain III (DIII) of the E protein. By deleting or substituting amino acids in the loop to mimic motifs present in other mosquito-borne or tick-borne flaviviruses, it was shown that retention of the DIII FG loop structure is important whereas the specific amino acid sequence can vary, providing the loop is maintained (Erb et al., 2010). These observations related to viral infection and replication in mammalian cells as well as in *Aedes aegypti* mosquito midguts, but not C6/36 cells where the FG loop was found to be dispensable. All the FG loop mutants were able to bind to and enter mammalian cells but replication of mutants mimicking the tick-borne FG-loop was delayed in Vero cells at 37 °C until secondary mutations appeared. These finding emphasize the importance of the E protein DIII FG loop structure in DENV infection and replication in mosquitoes and mammalians cells.

4.2.4. Protein modifications

4.2.4.1. NS1. glycosylation. In order to investigate the importance of NS1 protein glycosylations, site-directed mutagenesis of a full length infectious clone of DENV-2 was used to create mutant viruses lacking the NS1-130N, NS1-207N or both of these NS1 glycosylation sites (Crabtree et al., 2005). The results showed that viruses altered either at the Asn-130 or the Asn-207 position, exhibited reduced growth characteristics in C6/36 cells, reduced NS1 secretion from infected cells and decreased neurovirulence in a mouse model. Ablation of both NS1 glycosylation sites resulted in unstable viruses that acquired numerous additional mutations. Taken together, these results indicated that glycosylation of the DENV-2 virus NS1 protein may influence NS1 protein processing/transport as well as the pathogenicity of the virus.

4.2.4.2. Envelope glycosylation. Using an infectious clone derived from the WNV strain NY99, glycosylation within the E protein was shown to enhance neuroinvasiveness in a mouse virulence model (Beasley et al., 2005). The introduction of the prM-E genes of the strain ETH76a, which is attenuated for mouse neuroinvasion and possess the E-N154S mutation that abolished the glycosylation motif at residue E154, into an infectious clone of the highly neuroinvasive NY99 strain (E-154S in ETH76a and E-154N in NY99), or specific mutation of residue E154 to only abolish glycosylation, resulted in attenuation of that virus to a level comparable with ETH76a. Furthermore, a mutation that permitted glycosylation of the ETH76a E protein in the NY99 backbone yielded a virus with virulence equivalent to NY99. These results confirmed that the highly virulent mouse neuroinvasion phenotype of strain NY99 compared to the attenuated mouse neuroinvasion phenotype of strain ETH76a is primarily mediated via the presence of the E protein glycosylation motif.

4.2.4.3. Non-essential dimerisation of NS1. It was demonstrated in 1999, that a single amino acid substitution of a conserved proline residue in the NS1 protein of KUNV (P250L) results in loss of dimer formation, without inhibiting virus replication and the correct trafficking of this protein in infected cells (Hall et al., 1999). However, virus yields were reduced and its virulence diminished. Contrary to what was previously reported, these results demonstrated that dimerisation of the flavivirus NS1 protein is not an essential requirement for NS1 protein secretion, virus replication and neurovirulence in mice (Pryor and Wright, 1993, 1994; Pryor et al., 1998). These observations were confirmed when it was shown that a homologous substitution at residue 250 of MVEV NS1 protein also abolished NS1 dimerization but replication efficiency of the mutant virus was reduced in mammalian cells and neuroinvasiveness was reduced in weanling mice (Clark et al., 2007).

4.2.5. Cyclization sequences and RNA secondary structures

4.2.5.1. Cyclization sequences. Using atomic force microscopy and RNA binding assays, it was first shown that the known 5′ and 3′ cyclization sequences present in all mosquito-borne flaviviruses (MBFV), were essential for the formation of RNA–RNA complex formation (Alvarez et al., 2005b). However, additional complementary sequences present at the 5′ end immediately Upstream of the initiator AUG Region (UAR) and at the 3′ end within the 3′ SL (5′ and 3′ UAR) of the viral RNA were also shown to be required. In order to investigate the functional role of the 5′–3′ UAR, these sequences were mutated either separately, to destroy base pairing, or simultaneously, to restore complementarity in a DENV infectious clone. Non-viable viruses were recovered after transfection of DENV mutants carrying specific substitutions within the 5′ or 3′ UAR, while the mutant containing the compensatory mutations that restore complementarity was able to replicate demonstrating that base pairing of 5′–3′ UAR provides an essential element for viral viability and that cyclization of the RNA is a required conformation for viral replication (Alvarez et al., 2008).

4.2.5.2. Cis-acting element. Using structure prediction and biochemical analysis, Liu et al. (2013) identified a novel conserved *cis*-acting element, downstream of the 5′ cyclization sequence pseudoknot (DCS-PK), a three-stem pseudoknot structure located in the capsid-coding region of mosquito-borne flaviviruses (Liu et al., 2013). Mutagenesis of an infectious clone of DENV-4 revealed that disruption of the DCS-PK structure hindered the ability of 5′ RNA to bind to 3′ RNA, whereas restoration of the DCS-PK structure facilitated recovery of the 5′–3′ complex demonstrating its role in viral replication. Mutagenesis also showed that the function of DCS-PK depends on its position relative to the 5′ cyclization sequence, its secondary structure and its specific primary sequence. Based on these results, the authors proposed that the *cis*-acting element DCS-PK enhances viral replication by regulating genome cyclization, and that DCS-PK might interact with other *cis*-acting elements to form a functional viral RNA cyclization domain, thus playing a critical role during the flavivirus life cycle.

4.2.5.3. Replication enhancer element. A unique replication enhancer element (REE) was identified within the capsid gene of TBEV (Tuplin et al., 2011). Using thermodynamic and phylogenetic analyses, it was predicted that the REE folds as a long stable stem–loop structure (designated SL6) with an exposed conserved hexanucleotide among all tick-borne flaviviruses (TBFV). To investigate the role of SL6, a TBEV infectious clone was used to introduce nucleotide substitutions which altered either the linear sequence of the unpaired apical loop or destabilized the base-paired stem. The greatest phenotypic changes were observed in mutants with a destabilized stem while point mutations in the conserved hexanucleotide motif of the terminal loop caused moderate virus attenuation. However, the fact that all mutants eventually reached the titer of wild-type virus late post-infection demonstrated that the SL6 REE is not essential for growth in tissue culture but acts to up-regulate virus replication.

4.2.5.4. Cartography of the 3′ UTR. The study of flaviviral 3′ UTR sequences has attracted considerable interest and led to in depth *in silico* analyses (Gritsun and Gould, 2007; Gritsun et al., 1997, 2006). Likewise, infectious clones have been used for mapping, defining and characterizing the various component parts of the 3′ UTR. A series of progressive deletions was introduced into the 3′ UTR of a DENV-4 infectious clone and the viability of the resulting mutants was analyzed (Men et al., 1996). Deletion of the Conserved Sequence (CS) 1 in the 3′-distal region, which is highly conserved in the widely divergent MBFV group, completely abolished infectivity indicating that this sequence is essential for virus replication. In contrast, deletion of either the CS2-A or CS-2B or both (CS2), which are also conserved between all MBFV, was not lethal.

Likewise, progressive upstream deletions from the CS2 towards the stop codon yielded viable mutant viruses although they exhibited a spectrum of growth reduction in cell culture. Thus, it was proposed that the boundary between viable and lethal deletions lies immediately upstream of the CS1 as deletion mutants that retain only the 3′-distal regions CS1, SL2 and the terminal long stable hairpin (3′LSH) produced viable virus albeit with low infectivity and only in mosquito cells. For infectivity in vertebrate cells, the presence of CS2 was also essential. In general, the impact of deletions in 3′ UTRs downstream of CS1 was more profound in primate cell than in mosquito cells suggesting that host factors may play an important role in efficient viral replication (Men et al., 1996).

Similar results were produced using an infectious clone of YFV. The conserved sequence 5′ CS and CS1 as well as the 3′ stem loop structure were found to be essential for virus replication in cell culture whereas mutants with deletions in CS2 and the region containing the YFV-specific repeated sequences (between CS2 and the stop codon) still yielded viable viruses, although with delayed growth properties and smaller plaque size, suggesting that this region is not essential for virus viability but may have a function related to virus replication efficiency (Bredenbeek et al., 2003).

Deletions were also introduced in the 3′ UTR of the TBEV (Mandl et al., 1998) and the KUNV (Khromykh and Westaway, 1997). *In silico* analysis of the consequences of the introduction of deletions in DENV, TBEV and KUNV was conducted to evaluate their impact on secondary structures (Proutski et al., 1999).

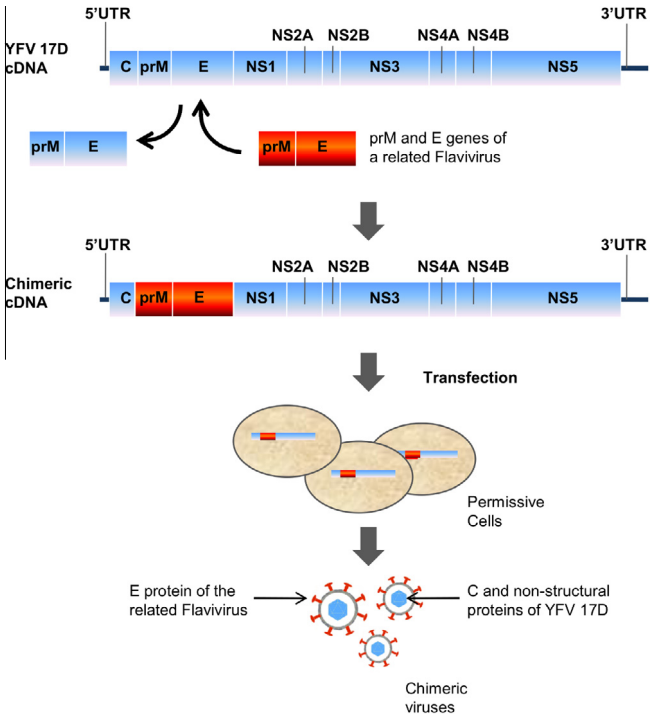

Fig. 6. Schematic representation of the general approach for the design of YFV 17D-based chimeric vaccine candidates. The sequences encoding the prM and E proteins of the vaccine target virus (e.g., JEV, WNV, DENV or SLEV) were engineered into the cDNA genome of the YFV 17D vaccine strain to replace the authentic prM and E sequences. Chimeric viral RNA were obtained by *in vitro* transcription and were transfected into permissive cells, resulting in the production of infectious chimeric virus particles that are covered by the vaccine target virus envelope proteins.

4.3. Reverse genetics methods as therapeutic tools

The development of reverse genetics methods made it possible to develop new approaches to the production of live attenuated vaccine candidates, (through the creation of chimeric viruses), the discovery and characterization of potential antiviral therapeutic molecules, and understanding the basis for the appearance of drug resistance.

4.3.1. Chimeric vaccines

The "chimeric" approach for vaccine design is based on the observation that structural genes of one flavivirus can be exchanged with equivalent genes from other related flaviviruses without significantly affecting the replicative capacity of the derived recombinant (chimeric) virus. For example the chimeric virus could contain structural genes from one flavivirus (e.g., prM and E protein) and the remaining parts of the genome (including the terminal untranslated regions) from another flavivirus to provide the "background" supporting genes which encode non-structural proteins to provide replicative and protein processing functions. The rationale behind this strategy is to confer long-lasting protective immunity against pathogens using a known attenuated virus to provide the backbone function for the immunizing antigens. The E and prM proteins contain critical antigenic determinants that induce protective neutralizing antibodies.

The feasibility of this approach was first demonstrated by Bray and Lai (1991), who constructed the first intertypic DENV chimeras (Bray and Lai, 1991). The entire structural region (C-prM-E genes) or only the envelope protein genes (prM-E) of a wild-type DENV-4 strain 814669, as well as the non-structural NS1 protein, were successfully replaced with the corresponding genes from, DENV-1, DENV-2 (Bray and Lai, 1991) (Bray et al., 1998), or DENV-3 (Chen et al., 1995). The chimeras grew in cell cultures, displayed the expected antigenic specificities, and elicited protective levels of expected neutralizing antibodies in monkeys (Bray et al., 1996).

These studies represent the foundation for the construction of a variety of chimeric flaviviruses that are being developed as potential live human vaccines. Due to its impressive safety record and high efficacy as a human vaccine, the attenuated YFV 17D strain has been used as the backbone for the construction of some of these chimeric vaccine viruses (see Fig. 6). First developed by Chambers et al. (1999) and Monath et al. (1999) for the generation of YFV/JEV chimera (Chambers et al., 1999; Monath et al., 1999), YFV-based chimeric candidate vaccines have been subsequently constructed for DENV (Caufour et al., 2001; Guirakhoo et al., 2000, 2001, 2006; van Der Most et al., 2000), JEV (Monath et al., 1999, 2003), WNV (Arroyo et al., 2004; Monath et al., 2001, 2006) and Saint Louis encephalitis virus (Pugachev et al., 2004) and have now been extensively tested in clinical trials with results that demonstrate their immunogenicity and good safety profile (Guy et al., 2008, 2010). Structural genes were either derived from wild type (WT) viruses (e.g., DENV and veterinary WNV vaccine candidates) or from empirically derived attenuated vaccines (e.g., JEV strain SA14-14-2) or by introduction of specific attenuating mutations into the wild-type E gene by site directed mutagenesis (e.g., NY99 strain for development of a human WNV vaccine). The JEV strain SA14-14-2 has also been used as the backbone to produce DENV-2/JEV, WNV/JEV or TBEV/JEV attenuated chimeras (Li et al., 2013a,b; Wang et al., 2014). A chimeric WNV vaccine candidate, WNV/DENV-4, was also reported (Maximova et al., 2014; Pletnev et al., 2002).

The issue of the possible occurrence of recombination between a chimeric virus and a WT flavivirus that could lead to the production of a recombinant virus with unanticipated properties has been raised and remains unanswered (Seligman and Gould, 2004).

4.3.2. Antiviral drugs

Infectious clones can be used to characterize antiviral drug resistance mechanisms. For example, resistant mutants obtained *in cellulo* by passaging the virus with increased concentrations of antiviral drug, can be characterized using reverse genetics methods with infectious clones. By introducing the candidate mutations into the genome of the infectious clone the resistance phenotype of each mutated virus can be determined. This approach was used to characterize the antiviral properties of brequinar (BQR) using DENV (Qing et al., 2010). After appropriate mutant selection in cell culture, followed by sequencing and appropriate mutagenesis of the infectious clones, it was demonstrated that a mutation in either the envelope or NS5 gene confers BQR resistance. Mechanistically, the envelope mutation renders the mutant virus less susceptible to BQR inhibition during the assembly/release phase whereas the NS5 mutation confers resistance through enhancement of viral RNA synthesis.

In another study, Yang et al. (2014) used a stable reporter-DENV replicon cell line to identify a small molecule inhibitor, BP13944, which specifically inhibit all four serotypes of DENV. Sequencing analyses of isolated resistant mutants identified a consensus amino acid substitution (E66G) present within the NS3 protease domain. Introduction of this mutation into a DENV-2 infectious clone resulted in the apparition of the resistance phenotype to BP13944. The location of the mutation, within the interface between the protease and helicase domains of the DENV NS3 protein, indicates that BP13944 may affect DENV replication by binding to the protease interface (Yang et al., 2014).

5. Conclusions and perspectives

The availability of cDNA clones/PCR-amplicons has facilitated the study of the genetic expression, replication and pathogenesis of RNA viruses using mutagenesis, deletions, and insertions and complementation experiments. It has also enhanced our understanding of the molecular mechanisms of natural or induced RNA recombination as well as virus interactions with host cells and organisms.

The production of infectious clones was an essential step in the development of reverse genetics systems and is now beginning to enable the acquisition of a huge amount of knowledge for these viruses. Democratisation of the technology for *de novo* synthesis and the huge developments, that have been made during recent decades, have made it possible to generate new investigative approaches that avoid the need to use critical steps for infectious clone construction (cloning, propagation of cDNA into bacteria or *in vitro* RNA transcription) thus providing easier, faster and more reliable methods with which to unravel the scientific mysteries discussed in this review. These methodologies will undoubtedly continue to improve and become even more accessible, potentially ensuring an increasingly exciting future for scientific investigation.

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