Generic RT-nested-PCR for detection of flaviviruses using degenerated primers and internal control followed by sequencing for specific identification

M.P. Sánchez-Seco, D. Rosario, C. Domingo, L. Hernández, K. Valdés, M.G. Guzmán, A. Tenorio

Abstract

Flaviviruses are a widespread and numerous group of arboviruses that can cause serious illness in humans. The continuous and slow spread of certain flaviviruses, such as Dengue viruses, and the recent entry and spread of West Nile virus to the American continent, point to the need to control these infections. This control requires the use of suitable techniques for diagnostic and surveillance programmes. A generic RT-nested-PCR that is, theoretically, able to detect each member of the group has been designed. The identification of the detected virus is carried out by sequencing. The introduction of an internal control would reduce the number of false negative results and could be used to quantify the viral load in clinical samples where the method works well.

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Keywords: Flavivirus; Generic PCR; Detection; Degenerated primers

1. Introduction

The genus Flavivirus, spread worldwide, belonging to the Flaviviridae family, is composed of a wide group of pathogenic viruses transmitted by arthropods. These are enveloped viruses, the genome of which is a single-stranded, positive polarity RNA molecule, of approximately 11 kb in length (Murphy et al., 1995).

At least 30 flaviviruses cause disease in humans. Most of these infections are asymptomatic, and disease may vary from a febrile illness with or without rash, to life-threatening conditions, such as hemorrhagic fever or encephalitis. Moreover, flaviviruses cause severe and economically significant diseases in domestic animals. Tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV), Saint Louis encephalitis virus (SLEV), Yellow fever virus (YFV), Dengue viruses, serotypes 1–4 (DENV1–4), or West Nile virus (WNV) are key human pathogens (Burke and Monath, 2001).

DENV causes more than 50 million cases of human infection worldwide each year, resulting in around 24,000 deaths (WHO, 1999; Gibbons and Vaughan, 2002). The disease ranges from a mild, febrile illness to severe hemorrhagic Dengue fever. DENV in endemic in the Americas, South-East Asia, the Western Pacific, Africa and the eastern Mediterranean region (WHO, 2000; Gubler, 2001). Yellow fever has gained considerable notoriety in the last few years, due to resurgence in epidemic activity in South America and Africa (Monath, 1997, 2001). Disease in travellers returning from endemic regions, caused by both viruses, has also been reported (Drosten et al., 2003; Stephan et al., 2002). WNV was...
introduced for the first time into New York, USA, during the summer of 1999 (Briese et al., 1999). Its vector has spread quickly, and during the following years the virus has invaded 46 states in the USA, causing more than 9000 human cases, which were fatal in 264 (Anonymous, 2003).

These viruses are a real concern for public health in developed countries, which are setting up surveillance programs for their rapid and reliable detection and identification. However, developing countries also require rapid, reliable and simple techniques for diagnosis.

Flaviviruses share common antigens, which make serology studies hard to interpret, and diagnosis based on this methodology difficult. Many of these viruses also share their transmission vectors, leading to frequent co-circulation of different viruses, and thus hampering precise identification (WHO, 1999).

Specific molecular diagnosis has been designed for some flaviviruses, and a number of attempts have also been made at generic detection (Chow et al., 1993; Fulop et al., 1993; Tanaka, 1993; Chang et al., 1994; Puri et al., 1994; Pierre et al., 1994; Meiyu et al., 1997; Kuno, 1998), although only one generic RT-heminested-PCR reaction with a high degree of sensitivity has been developed (Scaramonzzo et al., 2001; Lanciotti, 2003).

The aim of this study was to develop a sensitive and reliable technique for flavivirus detection and identification, to be used as a suitable diagnostic and surveillance tool. The introduction of an exogenous target provided us with an internal control (IC) for the extraction and amplification processes. The amplification of this internal control can also be measured and used to quantify the viral load.

2. Materials and methods

2.1. Viruses and their propagation

YFV, strain 17D, was obtained from Stamaril (Aventis Pasteur, Strasbourg, France).

Vero cell monolayers, maintained in MEM, supplemented with FBS 2% and with a mixture of penicillin and streptomycin 0.01% (Gibco BRL), were infected with YFV and cultured for 5 days, until a cytopathic effect was evident. Infected cultures were scraped subsequently and the infected cells harvested in MEM. Titration was undertaken following the protocol described by Morens et al. (1985).

2.2. Obtaining of RNAs

Viral RNA was extracted from the infected cell cultures and from clinical samples, following a procedure described previously (Casas et al., 1995). In brief, infected cells were disrupted and 50 µl of the supernatant fluids or clinical samples were incubated with 200 µl of a guanidinium thiocyanate lysis buffer, nucleic acid was precipitated with an equal volume of isopropanol and the pellet was dried out after washing with ethanol 70% and dissolved in 10 µl of ribonuclease-free water. Alternatively, QiAamp Viral RNA Mini Kit (Qiagen) was used following manufacturers indications.

RNAs from DENV1, Hawaii strain, DENV2, New Guinea C strain, DENV3, H87 strain, DENV4, H241 strain, JEV, Nakagana strain, TBEV, vaccine strain, SLEV, 52 strain, WNV, B956 strain, and Murray Valley Encephalitis Virus (MVEV), MV/1/1951 strain, were acquired commercially from the National Collection of Pathogenic Viruses (UK).

2.3. Generic oligonucleotides

Alignments were undertaken using nucleotide sequences of RNA of different flaviviruses, obtained from GenBank (National Institute of Health, Bethesda, MD, USA), using the MACAW 2.0.5 programme (Schuler et al., 1991). Table 1 shows the abbreviations and accession numbers for the viruses whose sequences have been used.

Degenerated primers were designed based on conserved motifs in a region of gene NS5, which encodes for the polymerase, to perfectly align with known flaviviruses sequences. Primers selected were:

Flavi1+: 5′ TGYTTGGITGGYGGIGGIRGITGG 7897 3′,

Flavi1−: 5′ TGYTTGGITGGYGGIGGIRGITGG 7897 3′,

Flavi2+: 5′ TGYRTIY A Y AWCA YSA TGGG 9006 3′,

Flavi2−: 5′ TGYRTIY A Y AWCA YSA TGGG 9006 3′,

The primers designated as Flavi1 were used in the RT-PCR amplification and Flavi2 in the nested amplification. The symbols (+) and (−) respond to sense and antisense sequences, respectively. Indicated positions correspond to those of YFV strain 17DD (accession number: U17066).

2.4. Generic RT-PCR

Reverse transcription of RNA to cDNA and subsequent amplification were carried out using the Access RT-PCR System (Promega, Madison, WI, USA) in a PCT-200, Peltier Thermal Cycler (MJ Research, Watertown, MA, USA) utilising thin-walled reaction tubes (REAL, Durviz, Valencia, Spain) with no mineral oil overlay. Briefly, 5 µl of nucleic acid preparation was added to 45 µl of a RT-PCR Mix containing 2 mM MgSO4, 0.2 mM of dNTPs, 40 pmoles of each primer (1+ and 1−), 5 U of avian myeloblastosis virus reverse transcriptase and 5 U of the thermostable Taq DNA polymerase from Thermus flavus.

Samples underwent an initial cycle at 38 °C for 45 min and 94 °C for 2 min, followed by further 40 PCR cycles at 94 °C for 30 s (denaturation), 47 °C for 1 min (annealing) and 68 °C for 1 min and 15 s (elongation). A final extension step was carried out at 68 °C for 5 min.

2.5. Generic nested PCR amplification

The nested PCR reaction mixture was carried out in a final volume of 50 µl and contained 2.5 mM MgCl2 (Perkin-
Table 1

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Elmer-Cetus, Norwalk, CT), 0.1 mM of each dNTP (Amer- sham Pharmacia Biotech, Sweden), 40 pmols of each primer (2+ and 2−), 2.5 U of AmpliTaq DNA Polymerase (Perkin- Elmer-Cetus, Norwalk, CT) and 1 μl of the product of the first amplification. The mix was then subjected to an initial denaturation stage (94 °C for 2 min) followed by 40 PCR cycles. Conditions used were similar to those described in the previous paragraph. Minor changes were introduced in elongation and final extension. The first was carried out at 72 °C for 15 s, and the final step also at this temperature for 5 min.

Upon completion of the amplifications, 10 μl of each reaction mixture was analysed by electrophoresis with a 2% agarose gel containing 0.5 μg/ml of ethidium bromide in TBE buffer gels. Products were visualised under UV light. A 1 Kb DNA ladder (Boehringer Mannheim) was included on each gel.

2.6. Construction of the internal control

The MIMIC approach (Siebert and Larrick, 1993) was modified to obtain an internal standard as previously described by Fedele et al. (2000). Briefly, two tailed primers were designed to amplify the heterologous gene coding for the Pseudorabies virus (PrV) polymerase. At its 3’ end the primer named MimFlavSen contains a sequence, which aligns with PrV, and at its 5’ end a sequence matching that of Flavi1+. The other tailer primer, MimFlavAnt, also contains a sequence of the heterologous gene at its 3’ end and sequence corresponding to Flavi1−, which follows that of Flavi2+. The other tailer primer, MimFlavAnt, also contains a sequence of the heterologous gene at its 3’ end and sequence corresponding to Flavi1−, which follows that of Flavi2− at the opposite end.

Tailed primers were as follows:

MimFlavSen:

5′- gacctaggttgtggatgcggtagctggtgtattcacatcacgatggg-3′

MimFlavAnt:

5′- tcccatccggctatatcgtcagcaacctgttcctcattgagaaatcc-3′

PrV sequences are written in lower case, Flavi2+ and − sequences are underlined, and Flavi1+ and − sequences are represented in bold type.

The product resulting from PrV amplification using these tailed primers contains the four recognition sites for the flavivirus generic primers (Fig. 1). The fragment amplified by these tailed primers was purified in agarose gels and cloned in the pCR 4-TOPO vector, as described in the following section.

2.7. Cloning

The DNA band, resulting from first generic amplification of YFV RNA, was excised from the agarose gel using a GeneClean kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions and ligated with the plasmid pGEM-T (Invitrogen, The Netherlands) following the T/A cloning strategy. The resulting plasmid was named pYFl. Epicurean Coli XLI-1 Blue electroporation-competent cells (Stratagene) were transformed with the ligation mix and plated onto L-agar containing ampicillin, IPTG and X-Gal (Sambrook et al., 1989). Colonies containing recombinant plasmids were identified by PCR using the flavivirus generic primers.

Fig. 1. Diagram showing the preparation and cloning of the internal control molecule. The primer MimFlavSen contains the sequences of Flavi1+ (dark box), Flavi2+ (grey box) and a fragment of PrV (white box). MimFlavAnt contains the sequences of Flavi1− (dark box), Flavi2− (grey box) and another fragment of PrV (white box). The fragment amplified by these primers, which contains the recognition sites for the primers used in the flaviviruses generic PCR, was cloned and used later as internal control.

To clone the IC, the band resulting from amplification of PrV by the tailed primers was excised from the agarose gels using the QIAquick Gel Extraction Kit (QIAGEN) and ligated with the HTP TOPO TA Cloning Kit (Invitrogen) on to the pCR 4-TOPO vector, following manufacturers’ instructions. One Shot TOP10 Competent Cells (Invitrogen) were used to transform the resulting plasmid. Colonies containing recombinant plasmids were identified as described in the previous paragraph. Purified plasmid was also digested with Pst I (Boehringer Mannheim).

2.8. Amplification with the internal control

Amplification was carried out under the same conditions previously described for the generic reaction. IC and pYFL were amplified in the same tube. After electrophoresis through a 3% agarose gel, the intensities of the two bands were determined by densitometry using 1D Manager Software (TDI, Spain).

2.9. Direct sequencing

Bands from the second round of generic amplification were purified using QIAquick PCR Purification Kit (Qiagen, Germany). Sequencing reactions on both strands were performed with the ABI Prism BigDye Terminator Cycle Sequencing v2.0 Ready Reaction (Applied Biosystems, Foster City, CA), and analysed using an ABI model 377 automated sequencer (Applied Biosystems, USA).

2.10. Handling of sequences

The sequences obtained were compared with those recorded in databases, in order to identify the detected agent and to study the level of homology. The multiple sequence alignment programme Clustal W (1.6 Version) was used to obtain an optimal sequence alignment file. A tree was built using the neighbour-joining method, which calculated bootstrap confidence values of 1000 bootstrapping trials using the MEGA2 program (Kumar et al., 2001).

2.11. Clinical samples

Samples of whole blood, taken from four Spanish travellers returning from Dengue endemic regions, were used to validate the method. DENV infection has been previously diagnosed in these samples using a RT-nested PCR protocol developed for the detection of the four serotypes of dengue virus in clinical samples (Domingo et al., 2004). Sample was mixed with the lysis buffer to which IC had been added to obtain a final concentration of 500 copies per 5 μl. Nucleic acids were obtained as previously described. Blood was also obtained from a YFV vaccinated subject 0, 4, 6, 8, and 15 days post-vaccination in order to control viremia levels.

3. Results

3.1. Generic amplification of vRNAs

Primer annealing temperatures and concentrations, thermocycling parameters, and each reaction component were standardised by experimentation. The conditions described in Section 2 are the ones selected; however, the reaction also works in a wide range of salt conditions and annealing temperatures (data not shown).

A band of the correct size (1385 bp) was obtained following RT-PCR amplification and also a correct one after the nested reaction (143 bp) with all flaviviruses tested: JEV, MVV, SLEV, TBEV, WNV, DENV1–4 and YFV (Fig. 2). The amplification is also specific, since no bands were obtained when RNA from a non-infected cell culture was used as target.

The sensitivity of the reaction was determined using the extracted RNA of serial dilutions of a YFV stock with 2 × 10^3 TCID₅₀ ml⁻¹. We were able to detect about 10 TCID₅₀ per tube using the nested reaction (Fig. 3A). The limit of detection, in terms of number of copies of cDNA, was also calculated. With this aim, the product of the first
amplification of YFV was cloned and serial dilutions of the linearised plasmid (pYF) were assayed. The limit of detection was about 1000 copies in the first reaction and about 10 copies in the second (Fig. 3B).

3.2. Internal control

Serial dilutions of target pYF/L DNA were co-amplified in the presence of 100 copies of IC per tube (Fig. 4A), which affords a broad range of linearity, as could be shown when the standard curve was constructed by plotting the target/IC signal ratio against the logarithm of the initial amount of the target in the reaction tube (not shown). The sensitivity of the reaction is slightly hampered when the competitor molecule is added, however this could be improved with minor modifications in the reaction, by doubling the concentration of primers and changing the annealing temperature to 40 °C for 4 min (Fig. 4B).

3.3. Clinical samples

Some clinical samples from Spanish patients were used to validate the methodology and confirm the suitability of the technique for use as a diagnostic tool (Fig. 5). Viremia was detected 6 days after vaccination with YFV vaccine. Positive results were also obtained in three patients with DENV infection (samples 2, 3, and 4). The inhibition of sample 1 is demonstrated by the lack of amplification of IC.

3.4. Identification and grouping of the sequences

Double-stranded DNA products amplified in the generic nested PCR were directly sequenced as described in Section 2. The sequences of RNAs from the controls used, those obtained from clinical samples and equivalent ones, selected from the viruses listed in Table 1, were aligned with the Clustal programme and analysed using MEGA software to construct a tree based on their relationships (Fig. 6). Two main groups were formed: the mosquito-borne and tick-borne viruses with Cell Fusing Agent virus (CFAV) as the out-group sequence. Sequences of viruses that belong to the same species group together with very high bootstrapping values.

4. Discussion

Techniques used for diagnostic and surveillance programmes must be rapid and simple, and should detect and identify a wide range of pathogens to a high level of sensitivity. Generic RT-nested-PCR, followed by sequencing, match all these requirements. For this reason a generic RT-nested-PCR for flavivirus detection has been developed. The method
is able to detect a wide spectrum of viruses, is sensitive and can be used for clinical samples.

None of the methods described for generic or specific detection of flaviviruses are able to detect every member of this genus with a suitable degree of sensitivity. Only one RT-heminested-PCR has been described that would detect each one of the flavivirus group members when the viral load is low (Scaramozzino et al., 2001; Lanciotti, 2003). Degenerated oligonucleotides have been designed in homologous regions of the conserved NS5 gene that encodes for the polymerase, one of the best preserved proteins in RNA viruses, since avoiding specific primer sequences is required to amplify a wide spectrum of RNA viruses (Sanchez-Seco et al., 2003). We have tried to avoid mismatches by studying a great number of sequences of different viruses and using degenerated primers with special care over the third position of the codons.

DENV1–4 (dengue subgroup), JEV, MVV, SLEV and WNV (Japanese encephalitis subgroup) and yellow fever subgroup (YFV), which belong to the mosquito-borne group, were assayed and all of them rendered positive amplification (Fig. 2). One virus (TBEV) belonging to the tick-borne group also rendered a correct band when assayed. The wide spectrum of viruses detected; the design of our degenerated oligonucleotides; their high level of sensitivity; and their suitability for use with clinical samples (Figs. 2–5) make this a very useful method for detection of both known and unknown flaviviruses. The amplified flavivirus is identified by...
Flaviviruses are distributed in both developed and developing countries, so the ability of different laboratories to access the reaction components must be taken into consideration. Consequently, the methodology used for their study must be simple and easily performable. Our RT-nested-PCR is suitable for use with a wide variety of buffers and temperatures, and the volume of the reaction and the primers concentrations are low, in order to minimise costs. Nested amplification requires no more equipment or expertise than the first RT-PCR. Care, however, must be taken to avoid contamination, using isolated locations if possible.

The procedure described could, therefore, be used for surveillance purposes in regions in which sophisticated technologies have not yet been implemented. Its design will permit development of reactions for specific amplification of certain flaviviruses to make their diagnosis easy. With the aim of specific detection of the members of this genus that are able to co-circulate and cause hemorrhagic fever symptoms, we have developed a reaction for the specific detection of DENV1-4 and YFV. In order to do this, the RT-PCR described in this work has been used, and the nested one has been transformed in a multiplex nested-PCR (Sanchez-Seco et al., submitted for publication).

Acknowledgements

We would like to thank the Biopolymers Unit for their technical assistance, Fiona Westbury for her assistance in the preparation of this manuscript and Dr. L.E. Martin Otero and his team from La Fábrica Nacional “La Marañosa” (Spanish Ministry of Defence). This work has been partially backed by grants from the Fondo de Investigaciones Sanitarias (FIS) (Spanish Ministry of Health) (FIS 98/0229) and the Spanish Ministry of Defence. Drs. Sanchez-Seco, Tenorio and Domingo are members of the Red EVITAR (Enfermedades Víricas Transmitidas por Artrópodos y Roedores) and Red RICET (Red de Investigación Cooperativa en Enfermedades Tropicales), groups founded by the FIS (G03/059 and C03/04, respectively).

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