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Journal of Virological Methods 128 (2005) 113–127
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Rapid subgroup identification of the flaviviruses using degenerate primer E-gene RT-PCR and site specific restriction enzyme analysis

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Received 13 May 2004; received in revised form 19 April 2005; accepted 20 April 2005
Available online 31 May 2005

Abstract

A simplified and rapid method for the diagnosis of all flaviviruses could provide an important tool for understanding their epidemiology. A protocol based on the use of degenerate nested oligonucleotide primers and RT-PCR was developed for the identification of flaviviruses. The primers were selected to flank the three E-gene markers that identify the viruses, giving DNA products of 971–986 (outer primers) and 859–884 bp (inner primers). Eighty five percent of E genes from flaviviruses representing most of the genus were specifically amplified, representing viruses from each of the 14 virus groups defined by the seventh International Committee for the Taxonomy of Viruses. Categorisation of the flavivirus cDNA products into the corresponding virus groups was undertaken through restriction enzyme analysis by defining conserved restriction sites common to related viruses in appropriate virus groups. Ninety percent of the known vector-borne flaviviruses with published full length E-gene sequences could be identified within 10 h.

Keywords: Flaviviruses; RT-PCR; Universal primers; Flavivirus identification; Degenerate primers; Restriction enzyme analysis; Conserved restriction sites

1. Introduction

The *Flavivirus* genus exemplifies two major issues of public health concern (1) rapid rates of evolution and (2) emergence of new viruses that threaten human populations. Rapidly evolving flaviviruses are typified by dengue viruses, the evolution of which closely parallels the explosive growth of human populations (Zanotto et al., 1996). These viruses are currently estimated to cause 100 million infections annually (Monath and Heinz, 1995). Emerging flavivirus diseases include the tick-borne virus Kyasanur Forest disease virus (KFDV), which first appeared in India in 1957 (Work and Trapido, 1957), and the mosquito-borne virus Rocio virus (ROCV), which emerged in Brazil in 1975 (Lopes et al., 1978a,b). Since 1978, at least 25 new flaviviruses have been identified (Monath and Heinz, 1995). A simple procedure for identifying any new flavivirus within the genus would provide a sound basis for its precise classification and would help to improve our understanding of the origin and spread of these viruses.

The primer-directed PCR method (Saiki et al., 1988) has enabled the universal amplification of the genus *Flavivirus*, using primers in the highly conserved NS5 gene and 3′ UTR (Kuno, 1998a). Specific RT-PCR amplification of flaviviruses has been demonstrated with Yellow fever virus (YFV), West Nile virus (WNV), Kunjin virus (KUNV), Murray Valley encephalitis virus (MVEV), Japanese encephalitis virus (JEV) and St. Louis encephalitis virus (SLEV) (Eldadah et al., 1991; Tanaka, 1993; Puri et al., 1994) and the specific amplification of each of the four dengue virus (DENV) serotypes (Deubel

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0166-0934/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2005.04.006

et al., 1990; Henchal et al., 1991; Blok et al., 1992; Tanaka, 1993).

Whilst considerable attention has been focussed on developing universal RT-PCR protocols, methods for the rapid identification of a broad range of flaviviruses are less well developed. The identification of different flavivirus species or groups following their RT-PCR amplification with oligonucleotide primers which lack species-specificity has been undertaken using dot blot or slot blot nucleic acid hybridisation for YFV, WNV, MVEV, JEV, SLEV and DENV (Deubel et al., 1990; Tanaka, 1993; Henchal et al., 1991; Puri et al., 1994; Brown et al., 1994; Pierre et al., 1994), restriction digestion (Eldadah et al., 1991), enzyme linked immunosorbent assay (ELISA; Chang et al., 1994) and partial DNA sequencing (Chow et al., 1993; Whitby et al., 1993; Gaunt et al., 1997; Kuno et al., 1998b; Billoir et al., 2000). The wealth of nucleotide sequence data available for the pathogenically important flaviviruses provides the potential to diagnose them using a "site-directed" restriction digest strategy.

We describe an RT-PCR diagnostic procedure capable of identifying most of the sequenced flaviviruses, within 10 h of commencing the experiment. The primers consisted of a set of nested degenerate oligonucleotides, i.e. primer mixes. They were tested against each of the 66 flaviviruses described by Calisher et al. (1989), as well as 4 additional flaviviruses, subsequently recognised (Kuno et al., 1998b). The primers amplified 85% of these viruses. The E gene was the focus for this investigation as the three E-gene markers capable of precisely identifying Louping ill virus (LIV) can also uniquely identify other flaviviruses (Shiu et al., 1991; Venugopal et al., 1992, 1994; Gao et al., 1993a,b; Gritsun et al., 1993; Marin et al., 1995a,b). The pentapeptide marker EHLPTA is conserved in the tick-borne encephalitis virus (TBEV) complex at amino acid position 212–217. A unique species-specific marker has been identified at position 238–243 and a subgroup specific marker, at position 333–337, delineates the strains and serotypes of TBEV, JEV and DENV (reviewed by Marin et al., 1995a). Rapid diagnosis of amplified cDNA was achieved by the identification of conserved restriction enzyme sites capable of defining viral subgroups within the flaviviruses sequenced to date. The application of this work provides the framework for classification and epidemiological studies of the flaviviruses.

2. Methods

2.1. Universal primer design

A set of universal flavivirus oligonucleotide primers was designed by identifying consensus sequences from an amino acid alignment of 123 flavivirus E-gene sequences comprising 22 recognised flaviviruses (available at http://evolve.zoo.ox.ac.uk; Zanotto et al., 1996) (Table 1). These primer sequences flanked the three genetic markers at amino acid positions 212–217, 238–243 and 333–337 (Fig. 1). No absolute nucleotide conservation was found. Absolute primer specificity for every sequence in the alignment was achieved by designing degenerate primers (Fig. 1). The oligonucleotide primers were designed as an external primer pair, designated Uni for and Uni rev and an internal primer pair, designated Uni2 for and Uni2 rev. The predicted cDNA fragment size using Uni2 primers is 871–874 bp for LIV, TBEV and other Mammalian tick-borne viruses, 859 bp for the seabird virus group, 881–884 bp for JEV, MVEV and Kunjin virus (KUNV), 872 bp for WNV, 862 bp for YFV, 860 bp for DENV3 and 866 bp for DENV1, DENV2 and DENV4 virus. The predicted cDNA fragment size using Uni primers is 971–986 bp for tick-borne virus sequences and 974–998 bp for mosquito-borne virus flaviviruses. The degenerate primers Uni for and Uni rev, are universal for both tick-borne and mosquito-borne viruses, and Uni2 for and Uni2 rev comprise separate degenerate tick-borne and mosquito-borne virus degenerate primers designed over the same genomic region. Primers were synthesised on an Applied Biosytems Inc. DNA synthesizer and also by Genosys, and Cruachem, UK.

Since the study was completed Apoi (APOIV), Rio Bravo (RBV) (Billoir et al., 2000), Tamana Bat (TABV) (de Lamballerie et al., 2002), Montana myotis leucoencephalitis virus (MMLV) (Charlier et al., 2002) and Modoc (MODV) (Leyssen et al., 2002) full length E-gene sequences have been published as part of a genome sequencing project and are acknowledged by (*) in subject titles herein.

2.2. Viruses

The flaviviruses used in this study are the 66 that were serologically classified by Calisher et al. (1989), with the addition of Iguape virus (IGUV) (Coimbra et al., 1993), Greek goat encephalitis virus (GGEV), Spanish sheep encephalitis virus (SSEV) (Marin et al., 1995b) and Turkish sheep encephalitis virus (TSEV) (Gao et al., 1993a). Table 1 describes the full list of flavivirus species used in this study, and distinguishes between viruses with published E-gene sequence at the time of the study and viruses with no reported sequence data at the time of the study. Kunjin virus was not used in this study, instead a strain of WNV showing close sequence homology to KUNV was used (Gaunt et al., 2001). The viruses were either in the collection of The Institute of Virology and Environmental Microbiology (CEH, Oxford, UK) or were kindly supplied by Dr. Robert Shope (University of Texas, USA) and Dr. James Porterfield (University of Oxford, UK). All viruses were maintained as 10–20% suckling mouse brain suspensions (SMB), with the exception of KFDV. The KFDV E gene used in the study had been cloned into a baculovirus vector and expressed in *Spodoptera frugiperda* (*Sf*) cells and was kindly supplied by Dr. K. Venugopal (IAH, Compton, UK).