Molecular detection and typing of human picornaviruses

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Abstract

Picornaviruses include several important clinical pathogens which cause diseases varying from common cold to poliomyelitis and hepatitis. Introduction of RT-PCR methods for the detection of these viruses has significantly facilitated the diagnosis of picornavirus infections and elucidated their etiological role in clinical illnesses. Partial sequence analysis of the genomes has been used for typing of the viruses and in studies of molecular epidemiology of picornaviruses. These molecular approaches are likely to become the most predominant techniques for the diagnosis and epidemiological analysis, particularly in the enterovirus infections. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

In the Picornaviridae family, enteroviruses, hepatoviruses, parechoviruses, and rhinoviruses include human pathogens while aphtho- and cardioviruses cause disease in animals. Based on pathogenicity in experimental animals, human enteroviruses have been subgrouped into polioviruses (PV; 3 serotypes), coxsackie A viruses (CAV; 23 serotypes), coxsackie B viruses (CBV; 6 serotypes), echoviruses (EV; 28 serotypes), and enteroviruses 68–71. The outcome of enterovirus infections in man varies from minor respiratory illnesses to paralysis, myocarditis, meningoencephalitis and generalized infections with multiorgan failure in newborns (Grist et al., 1978). Most of the enterovirus infections, however, are assumed to be asymptomatic. Although several enterovirus serotypes can cause many of the illnesses mentioned, there are disease entities that are typically caused by one or only a few serotypes. These include epidemic poliomyelitis (PVs), hand-foot-and-mouth disease (mainly CAV16), acute hemorrhagic conjunctivitis (CAV24 and enterovirus 70), herpangina (certain CAVs) and pleurodynia (CBVs) (Grist et al., 1978).

Over 100 human rhinovirus (HRV) serotypes have been identified. They are considered to be the most frequent cause of the common cold. Moreover, HRV infections may result in sinusitis, otitis media, or exacerbation of asthma (Pitkäranta and Hayden, 1998). Due to its unique pathogenicity and genetic distance when compared to other picornaviruses, hepatitis A virus (HAV), previously designated as enterovirus 72,

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was reclassified in hepatovirus genus (Minor, 1991). More recently, two echoviruses (serotypes 22 and 23) with exceptional molecular properties have been assigned to a new genus, parechovirus (Hyypiä et al., 1992; Stanway et al., 1994; Mayo and Pringle, 1998). Examples of clinical diseases associated with picornavirus infections are shown in Table 1.

Conventional diagnostic methods for human picornavirus infections include culturing of the virus in susceptible cell lines, followed by neutralization typing (enteroviruses, parechoviruses) or acid lability testing (HRVs). Serological detection of enterovirus-specific antibodies provides indirect evidence of the etiology of the disease. Diagnosis of HAV infections is based on demonstration of specific IgM-class antibodies. In general, the isolation methods currently used for picornavirus identification are cumbersome, laborious and insensitive. Even at its best, several days are required for specific diagnosis. Hence, the introduction of newly developed molecular techniques into picornavirus diagnosis that provide means for faster and more sensitive virus detection has been highly appreciated. These methods improve not only laboratory diagnosis and clinical management of picornavirus infections, but increase

Table 1
Diseases associated with human picornavirus infections

Disease	Virus
Poliomyelitis	Polioviruses 1, 2 and 3
Paralytic disease	Coxsackievirus A7, enterovirus 70 and 71
Meningitis/encephalitis	Several enterovirus serotypes
Myocarditis	Coxsackie B viruses
Neonatal infections	Coxsackie B viruses, echoviruses
Pleurodynia	Coxsackie B viruses
Herpangina	Coxsackie A viruses
Hand-foot-and-mouth disease	Coxsackievirus A16, enterovirus 71
Acute hemorrhagic conjunctivitis	Coxsackievirus A24 and enterovirus 70
Respiratory infections	Many enteroviruses, parechoviruses
Common cold	Rhinoviruses
Gastroenteritis	Parechovirus 1
Acute hepatitis	Hepatitis A virus

knowledge of the clinical importance of human picornaviruses. The authors summarize here recent advances in RT-PCR and sequence analysis of human picornaviruses which, together with in situ hybridization techniques, have significantly improved the virological laboratory diagnosis of these infections.

2. Detection of picornaviruses by RT-PCR

Sequence analysis and the use of cDNA clones for the detection of several enterovirus serotypes by one single probe had suggested that some genome regions are highly similar among members of the genus. Moreover, HRVs were shown to be closely related to human enteroviruses. Based on these observations, attempts were made to select primers and probes which could be used for the development of RT-PCR assays for these virus groups.

The picornavirus genome is an $\approx 7000–9000$ nucleotides (nt) long, single-stranded RNA molecule. The long open reading frame, coding for the capsid and nonstructural proteins, is preceded by a 5′ noncoding region (5′NCR; 750 nt in enteroviruses). This region contains several conserved motifs which participate in the formation of secondary structures required for translation and replication of the virus genome. Most of the RT-PCR assays described have taken advantage of these homologous regions which are shared by entero- and rhinoviruses (Fig. 1). Because the amplification products are usually of a similar length, a specific oligonucleotide probe is needed for discrimination between the two picornavirus groups (Hyypiä et al., 1989; Santti et al., 1997). Alternatively, a primer may be targeted to a conserved region in the VP2 capsid protein gene and used together with another primer from the 5′NCR (Olive et al., 1990). An advantage with this primer pair is that the entero- and rhinovirus amplicons differ in length, due to an apparent deletion in the HRV genome in the region preceding the initiation codon. It has been shown in a number of studies that virtually all the enterovirus serotypes and most of the HRV isolates can be detected using these primer sequences (Hyypiä et
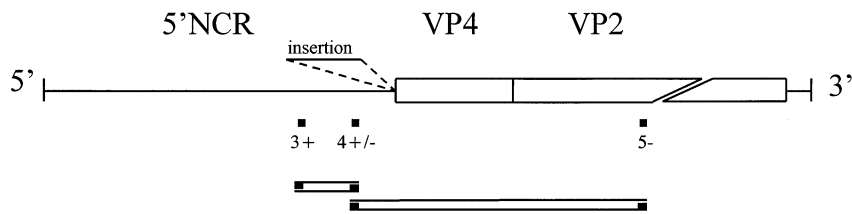
Fig. 1. Schematic representation of the picornavirus genome and location of commonly used RT-PCR primers for the detection of entero- and rhinoviruses. The apparent insertion of ≈ 120 nucleotides seen in the enterovirus genome when compared to rhinoviruses is indicated.

al., 1989; Horsnell et al., 1995; Pulli et al., 1995; Arola et al., 1996; Huttunen et al., 1996; Pitkäranta et al., 1997; Hyypiä et al., 1998; Oberste et al., 1998)

The authors became convinced of the usefulness of this technique during a recent outbreak of aseptic meningitis in Finland. The first cases of the illness, subsequently shown to be caused by echovirus 30, were diagnosed in the late summer 1996 and the peak of the epidemic occurred in September and October. Specimens including CSF, throat swabs, stool samples and sera were collected from the first eight patients with typical symptoms. The etiological agent was identified as an enterovirus from CSF by RT-PCR (Arola et al., 1996), after which this technique was mainly used for virological diagnosis. RT-PCR was positive from seven of the eight CSF samples, while virus isolation was positive from three CSF, throat swabs and stool specimens, all from the same three patients (Table 2). RT-PCR from serum samples gave positive diagnosis in five patients including one patient whose CSF remained negative in the RT-PCR test. Enterovirus IgM was positive in four sera by enzyme immunoassay.

Microbial etiology of the common cold in young adults was recently examined using conventional diagnostic methods and HRV RT-PCR by Mäkelä et al. (1998). HRVs were demonstrated in 53%, coronavirus infections in 9% and other viruses in 13% of the patients. Bacterial infections were rare and found in $< 5\%$ of these 200 patients with signs and symptoms of the disease. When virus isolation was compared with RT-PCR, the sensitivities in the detection of HRVs were 66 and 98%, respectively (Hyypiä et al.,

1998). HRV RNA has also been detected in maxillary aspirates and nasal swabs of patients with sinusitis (Pitkäranta et al., 1997).

During one epidemic year (July 1997–June 1998) 51 enteroviruses and 28 HRVs were detected from a total of 435 samples by using an RT-PCR assay at the Department of Virology, University of Turku. Enteroviruses were identified in 24 (18%) of the 135 CSF specimens analyzed while most of the other positive findings were from nasopharyngeal samples. The assay utilizes common 5′NCR primers and specific probes for entero- and rhinoviruses (Halonen et al., 1995). Thirteen samples, positive by RT-PCR based on the detection of an amplicon of the expected size in agarose gel electrophoresis, were negative in the hybridization assay, indicating need for further improvements in the reactivity of the probes.

Table 2
Virological findings in specimens from the first eight patients of a meningitis outbreak in Salo, Finland in 1996

Patient	CSF		Serum
	PCR	Virus isolation	PCR
1	+	−	−
2	+	−	+
3	+	EV30[a]	+
4	+	−	+
5	+	EV30	−
6	+	−	−
7	+	EV30	+
8	−	−	+

[a] EV30, echovirus 30.

3. Sequence analysis

Analysis of enterovirus genomes has shown that members of the genus form four genetic clusters when the coding region is used in comparisons (Fig. 2): cluster A includes some CAV serotypes and enterovirus 71; cluster B contains CAV9, CBVs and EVs; polioviruses (cluster C1) and certain CAVs (cluster C2) are closely related; while enterovirus 70 is a representative of cluster D [for review, see Hyypiä et al. (1997)]. Partial sequence analysis of virtually all enterovirus serotypes has shown that they belong to these four clusters (Pulli et al., 1995; Huttunen et al., 1996) and that the VP4/VP2 region sequence can be used for molecular typing of clinical isolates (Arola et al., 1996). It is notable, however, that, for instance, polioviruses cannot be discriminated from CAVs in the C cluster by this means and other genomic regions are required for the purpose. In the 5′NCR the clustering of enterovirus serotypes is different and does not provide a useful tool for typing, but can be used in some epidemiological studies. Although genetic clustering can also be detected among HRVs (Horsnell et al., 1995), the correlation of this grouping with pathogenesis of infection is not currently clear.

Partial sequence analysis of clinical isolates has been extensively used in the epidemiologic studies of polioviruses and resulted in a large sequence database (Rico-Hesse et al., 1987; Zheng et al., 1993; Huovilainen et al., 1995; Kew et al., 1995; Mulders et al., 1995; Chezzi et al., 1997; Morvan et al., 1997; Fiore et al., 1998). The global eradication program has significantly benefitted from these studies. A region of 150 nt in the junction of the structural gene VP1 and the viral protease gene 2A has been universally adopted for this purpose. The term genotype, which is defined as a cluster of genetically related viruses with < 15% nucleotide sequence divergence, has been used for poliovirus strains (Rico-Hesse et al., 1987). Within each serotype, the independent genotypes usually have distinct geographic distribution, consistent with the existence of separate foci of endemicity. These studies have provided an invaluable insight into the pathways of virus transmission and epidemiologic links.

In addition to polioviruses, several other enteroviruses have also been subjected to detailed epidemiologic analysis. These include the two serotypes responsible for large outbreaks of acute hemorrhagic conjunctivitis, CAV24 variant (CAV24v; Ishiko et al., 1992) and enterovirus 70 (Takeda et al., 1994). Regression analysis of the genetic distances between isolates has enabled estimation of the time of emergence of these viruses. The viruses were predicted to originate from single focal places; CAV24v in November 1963 $\pm$ 21 months, about 7 years before the first isolation of the virus in Singapore, and enterovirus 70 in August 1967 $\pm$ 15 months, 2 years before recognition of the virus in a pandemic of acute hemorrhagic conjunctivitis. The molecular epidemiology of CAV9, CBV1 and CBV5 as well as echovirus 30 which is characteristically involved in epidemics of meningitis has also been studied (Chang et al., 1992; Zoll et al., 1994; Kopecka et al., 1995; Gjøen et al., 1996). In hepatitis A virus infections, the molecular epidemiology has been investigated by many research groups and an extensive analysis of partial sequences from different geographic locations has been used to establish a useful database for further studies (Robertson et al., 1992). Recently, an RT-PCR assay was also developed for the detection of parechovirus 1 in clinical samples and the amplicons, representing the 5′NCR, were used in epidemiological analysis (Joki-Korpela and Hyypiä, 1998).

4. Conclusions

Increased knowledge of the molecular biology of picornaviruses and advances in laboratory techniques have allowed the development of RT-PCR assays which are more sensitive and rapid than the previously used isolation methods in the detection of entero- and rhinoviruses in clinical samples. Moreover, RT-PCR products can be used for typing and molecular epidemiological analysis of the viruses. The availability of RT-PCR assays has also enabled analysis of the presence of enteroviruses in various acute and chronic disease conditions, such as myocarditis and cardiomyopathies, central nervous system infections,
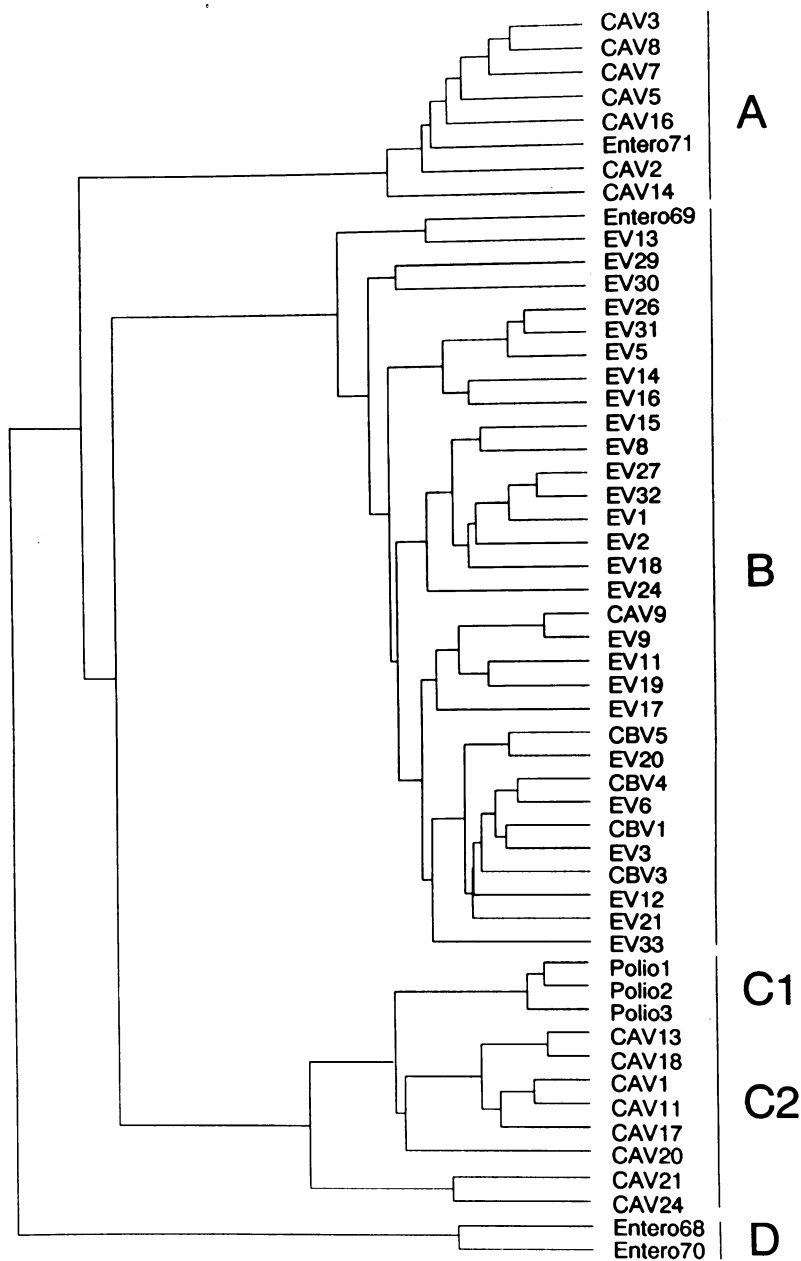
Fig. 2. Dendrogram based on partial sequences from VP2 capsid proteins shows genetic relationships between human enterovirus serotypes. Molecular clusters (A, B, C and D; Hyypiä et al., 1997) are indicated. The dendrogram was generated by using the PILEUP program from the Genetics Computer Group software (Devereux et al., 1984).

generalized neonatal infections, insulin-dependent diabetes mellitus and chronic fatigue syndrome.

Information on different picornavirus geno-

types will rapidly increase and, together with advances in mapping of pathogenic and antigenic determinants, this may allow more precise molec-

ular grouping of picornaviruses. It is possible that deduction of phenotypic characteristics from the genomic sequence may become possible in the near future.

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