Classification of enteroviruses based on molecular and biological properties

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Introduction

Classification of viruses into families is mainly based on virion morphology, the nature of the genomic nucleic acid and replication strategy. Other biological, physicochemical and antigenic properties are also in use and are important in identification and grouping of individual virus types and strains within the families. Classification of viruses has implications for two different communities whose aims may overlap but possibly remain distinct. For the scientist, classification and subgrouping of viruses should provide the framework for a better understanding of the biological properties of the groups thus defined. For the clinician, classification should provide a reliable basis for medically significant differentiation which will enhance diagnostic procedures and epidemiological studies as well as facilitate treatment and prevention of disease. The introduction of diagnostic procedures based on nucleic acid sequences, in particular PCR, has recently increased the need for precise classification of viruses in accordance with their molecular characteristics.

A further aspect is a view on contemporary generation of additional divergence, i.e. patterns of virus evolution. Different phenotypic properties of the virus can be used for classification in this context but if our aim is to understand the biological significance of current similarities and differences, we might prefer a parameter reflecting the history of generation of the divergence. Usually, it is assumed that members of a given virus group have a common ancestor. Hence, from this point of view, phylogenetic analysis of genomic nucleic acid sequences is likely to be the most useful basis for classification.

Enteroviruses form one genus of the Picornaviridae, a family of small, non-enveloped, positive-strand RNA viruses. The other picornavirus genera are aphthoviruses, cardioviruses, hepatoviruses and rhinoviruses. Genera are defined largely on the basis of physicochemical properties, such as virion density and acid sensitivity. Sixty-six distinct human enterovirus serotypes exist and this genus has been further divided into subgroups mainly on the basis of pathogenicity in experimental animals. Enteroviruses of lower animals have generally been classified by host species, e.g. porcine enteroviruses.

The purpose of this review is to show how the molecular and biological data now available are revealing consistent relationships between serotypes which offer an alternative to the current classification and enable more efficient diagnostic strategies. It will become clear that the current classification is not ideal and a molecularly-based scheme could accommodate many improvements.

Historical aspects

Landsteiner & Popper (1908) managed to transmit poliomyelitis into monkeys by using a filtrate, thus showing that the disease is caused by a virus. Later, it was observed that three different poliovirus serotypes existed, since no single antiserum was able to neutralize infectivity of all the strains studied. These were the first viruses to be propagated in tissue culture by Enders et al. (1949): a break-through in virology which led to the development of formalin-inactivated vaccines by Salk (1953) and live-attenuated vaccines by Sabin (1955). These vaccines are safe and effective, and have made possible the WHO goal of global eradication of paralytic poliomyelitis by the year 2000. The target of this campaign will be the elimination of the first identified and, in clinical terms the most serious, of the enteroviruses.

The availability of antisera against the three established poliovirus serotypes made it possible to study whether related, but antigenically distinct viruses existed. Dalldorf & Sickles (1948) isolated previously unrecognized viruses from faecal specimens of two children during a poliomyelitis outbreak. These pathogens induced paralysis in newborn mice, in contrast to polioviruses which usually cause disease only in primates. The viruses were called coxsackie viruses (later coxsackie A viruses; CAVs) referring to the first geographical site of isolation. CAVs induced flaccid paralysis which was different from the spastic paralysis observed after inoculation of the mice with certain other virus strains, today known as...
coxsackie B viruses (CBVs). Further studies have revealed that in experimental animals CAVs affect skeletal and heart muscle, while CBVs replicate in a wide range of tissues including the central nervous system, liver, exocrine pancreas, brown fat and striated muscle. Twenty-three CAV serotypes [1–22 and 24; CAV-23 was reclassified as echovirus (EV) 9] and six CBV serotypes (1–6) have been recognized.

The introduction of cell culture techniques into virus laboratories enabled the isolation of viruses which did not replicate in experimental animals. Echoviruses were isolated from stool samples, where polioviruses and coxsackieviruses are usually also found, but did not have the pathogenic properties of these subgroups in experimental animals; the name echovirus was chosen because their association with human disease was, at the time of their discovery, unknown (ECHO: enteric, cytopathogenic, human, orphan = no associated disease). It was originally postulated that whenever an echovirus was established as the aetiological agent of a clinically distinct disease, it would be removed from the group (Committee on the ECHO Viruses, 1955). However, it soon became evident that individual serotypes cannot be directly associated with individual illnesses but rather with a wide range of clinical manifestations. Therefore echoviruses are still classified together forming the largest enterovirus subgroup which consists of serotypes 1–7, 9, 11–27 and 29–33 (types 1 and 8 represent the same serotype, type 10 was shown to be a reovirus, type 28 was found to be a rhinovirus and EV-34 was shown to be a variant of CAV-24).

Thus the major characteristics used in subgrouping human enteroviruses are pathogenicity in man and in mice. Since the latter are not natural hosts for human enteroviruses, and since differences in pathogenicity may depend upon a limited number of molecular differences which may vary between isolates of a given serotype, it is not surprising that subsequent analysis indicates that the phylogenetic basis for the subgroups in some cases is tenuous. Indeed, it has been known for many years that different strains of an enterovirus serotype may exhibit different pathogenicities in the mouse system, thus confusing their subgrouping. Because of frequent difficulties in assigning enteroviruses to subgroups, more recently identified human enteroviruses have been merely numbered in their order of identification as serologically distinct new isolates (enteroviruses 68–71). Hepatitis A virus (HAV), previously classified as enterovirus 72, has been reclassified to the genus Hepatovirus of the Picornaviridae.

In the early 1950s, the use of monkey kidney cell cultures for the growth of polioviruses revealed the presence of latent simian viruses. Further investigations showed some of these to have the properties of enteroviruses and eventually 18 serotypes were described (Kalter et al., 1980). Investigations of viruses infecting domesticated animals in the late 1950s revealed the presence of enteroviruses in both pigs (Sus scrofa) and cattle (Bos taurus). To date, at least 13 porcine enterovirus (PEV) serotypes (Knowles et al., 1979; Honda et al., 1990; Auerbach et al., 1994) and two (or possibly three) bovine enterovirus (BEV) serotypes (Knowles & Barnett, 1985; Urakawa & Shingu, 1987) have been described. Enteroviruses isolated from African buffalo (Syncerus caffer), water buffalo (Bubalus bubalis), sheep (Ovis aries), goat (Capra hircus), deer (Cervus nippon) and impala (Aepyceros melampus) have been shown to be related to BEV-1 (Hamblin et al., 1985; Sharma et al., 1986; Urakawa & Shingu, 1987).

Implicit in the history of enterovirus classification is the existence of antigenically distinct viruses which can be considered to constitute serotypes. An operational definition for a serotype includes the idea that a strain represents a new serotype if it is not neutralized to a significant extent by antisera to previously characterized viruses and if it is not able to induce significant levels of neutralizing antibodies to these viruses (Committee on Enteroviruses, 1962). Unfortunately, in practice, some virus serotypes can tend to include strains representing an antigenic continuum which complicates the use of reference antisera. This can lead to difficulty in serotyping some isolates due to poor recognition by reference antisera or, alternatively, the observation of extensive cross-reactivity between some serotypes. The latter is most frequent between certain echoviruses, in particular EV-1 and EV-8, recently reclassified as a single serotype. Since current enterovirus identification is primarily based on serotypic differentiation, these difficulties can be significant for epidemiology and diagnosis. On the other hand, distinction between the three serotypes of poliovirus is clear, and inclusion of one representative of each serotype is necessary and sufficient in poliovirus vaccines.

Enterovirus diseases

One of the most distinctive diseases produced by an enterovirus is poliomyelitis. This is almost invariably caused by one of the three poliovirus serotypes, although several other enteroviruses have been reported to cause sporadic cases and even small epidemics of paralytic disease. ‘Typical disease’, paralytic poliomyelitis persisting for more than 2 months, is, however, a consequence of only a small minority of poliovirus infections, perhaps 1 in 100 to 1 in 1000 depending on the serotype and strain. Polioviruses may also cause aseptic meningitis or nonspecific minor illness, while in most cases infection is asymptomatic.

Clinical entities typically associated with particular enterovirus serotypes are otherwise rare although there are defined diseases which are usually caused by a limited number of different enterovirus serotypes. One of the most distinct examples is hand-foot-and-mouth disease (HFMD), a febrile mucocutaneous eruption that is most frequently associated with a coxsackievirus A16 infection. However, other CAV serotypes and enterovirus 71 have been reported to cause HFMD and CAV-16 has also been associated with other types of disease.

Other illnesses caused by enteroviruses include common
cold and other respiratory infections, rash, conjunctivitis, carditis, generalized infections of newborns and involvement of the central nervous system varying from mild meningeal symptoms to fatal cases of encephalitis and paralysis. In most cases of enterovirus disease, an aetiological diagnosis based on clinical symptoms only is impossible and members of several other virus families have also to be considered in addition to the almost 70 different enteroviruses. It is therefore obvious that disease pattern in humans is not a suitable criterion for serotype-based subgrouping of enteroviruses.

Enteroviruses have been associated with a number of disease conditions of domesticated animals (e.g., diarrhoea, reproductive failure). Two serotypes have been shown to be the causative agents of important diseases in pigs. Pig polioencephalomyelitis (Teschen disease), known since the 1930s, is caused by PEV-1 (and possibly by serotypes 2 and 3) and swine vesicular disease, first recognized in 1966, is caused by SVD virus (SVDV). The latter virus has been shown to be a porcine variant of human CBV-5 (Zhang et al., 1993).

**Molecular properties of enteroviruses**

**Virion structure and antigenic sites**

Since enteroviruses are small, nonenveloped icosahedral particles with a diameter of about 30 nm they were among the first human viruses to be studied in great detail using X-ray crystallography. A decade ago, the three-dimensional structure of poliovirus 1 (Hogle et al., 1985) was determined at a resolution which is sufficient to reveal the relative position of each individual atom in the virus capsid. The picornavirus capsid is made up of 60 identical building units, each containing one copy of the four structural proteins VP1–VP4. The folding pattern of polypeptides VP1–VP3 is similar, resulting in an eight-stranded antiparallel β-barrel structure. Surprisingly little difference is seen between type 1 and type 3 polioviruses (Filman et al., 1989) and structural differences between CBV-3 (Muckelbauer et al., 1995) and the two polioviruses are also located primarily on the virion surface. BEV also shares the overall architecture found in human enteroviruses, but the capsid has a relatively smooth appearance due to differences in surface projections (Smyth et al., 1995).

It is not clear whether significant differences exist between representatives of other enterovirus subgroups as the structures are not known yet. Alignment of primary sequences has, however, suggested that the main differences are found in the loop regions connecting the β-sheets (Pöyry et al., 1994). These are also the principal locations of antigenic sites in polioviruses (Minor et al., 1986). Originally, it was thought that there is a difference between the relative importance of distinct antigenic sites as regards poliovirus serotypes 1 and 3 (Minor et al., 1986) but more recent assessments of the location of the antigenic sites (reviewed by Mateu, 1995) do not fully support this view. Currently, the molecular counterparts of serotype definition in the capsid structure remain obscure.

**Replication cycle**

The first step in virus replication is the attachment of the virus to its cellular receptor, a cell surface molecule which the virus has become adapted to use in its entry into the cell. Capsid structure reorganization, brought about by the interaction with the cellular receptor(s), leads to release of the internal VP4 polypeptide and, subsequently, the RNA genome. The genomic RNA, approximately 7500 nt in length, is of positive polarity. It acts as an mRNA giving rise to a large polyprotein (200 kDa) which is autocatalytically cleaved by two viral proteases: 2A catalyses the cleavage between VP1/2A while 3C, sometimes in the form of its precursor 3CD, is responsible for the other cleavages. The activity responsible for the final maturation cleavage between VP4/VP2, which occurs after assembly of virus particles, is at present unknown.

The polypeptide-encoding region is preceded by a long untranslated region (5’UTR; ca. 750 nt) and followed by a shorter (70–100 nt) 3’UTR and a poly(A) tract. Replication of the genome is carried out by the RNA-dependent RNA-polymerase (3D) with the aid of other viral and host factors. Initially, a negative-strand copy is synthesized which is then used as a template for new genomic RNA-strands. A small polypeptide (VPg) is associated with the 5’ end of the genome and it may participate in replication and the assembly process, where the genomic RNA is packed inside the capsid. During infection, cellular protein synthesis is inhibited (host-cell shut-off) by the action of the 2A protease, which causes the cleavage of one of the translation initiation factors. This does not, however, inhibit viral protein synthesis since picornaviruses use cap-independent initiation in their own protein synthesis. Approximately 10^4–10^6 infectious virus particles are produced in one infected cell which is finally destroyed allowing the viruses to be released to infect new target cells.

All the details of the replication cycle have been uncovered using poliovirus type 1/Mahoney as the model virus. Differences between the enterovirus subgroups are not known in detail.

**Receptors**

Receptor specificity is a molecularly definable criterion which could be used in grouping enteroviruses. However, now that receptors for several enteroviruses are known, the molecular interactions on the cell surface have turned out to be more complex than expected. This is illustrated by poliovirus–receptor interactions. A few years ago, a member of the immunoglobulin superfamily was identified as the cell surface receptor for poliovirus (Mendelsohn et al., 1989). Since then, much has been learned about the mechanisms by which the virus recognizes this molecule. However, the receptor is expressed in a wide range of tissues, including those that the virus does not infect in humans. Recent data suggests that another protein, CD44, one of the lymphocyte homing receptors, expressed in tissues where poliovirus is known to
replicate, plays a role in the interactions thus determining or at least contributing to tissue tropism (Shepley & Racaniello, 1994).

Cell surface proteins belonging to the integrin family are recognized by some enteroviruses: \( \alpha_5\beta_3 \) is involved in cellular interactions of CAV-9 (Roivainen et al., 1994) but an alternative, as yet unidentified receptor also plays a role, at least in certain cell systems (Roivainen et al., 1991; Hughes et al., 1995). EV-22, a picornavirus which is only distantly related to CAV-9 in molecular terms (see below), also contains a functional RGD motif (Stanway et al., 1994) and these two viruses compete for cell surface binding (Roivainen et al., 1994). Interestingly, foot-and-mouth disease virus (FMDV), a member of the genus Aphthovirus, is also able to utilize DAF in their cellular interactions (Bergelson et al., 1995; Shafren et al., 1995). CAV-13, 15, 18, 20 and 21 are known to recognize the ICAM-1 molecule on the cell surface (Colonno, 1987; Pulli et al., 1995) thus competing for receptor binding with the members of the major rhinovirus group (Greve et al., 1989; Staunton et al., 1989).

In conclusion, receptor specificity might be a good candidate to complement the current classification based on pathogenic properties. However, at present too little is known about virus–cell interactions in different tissues to make these useful criteria.

Genetic clusters

To date, the complete nucleotide sequences of at least 19 human enterovirus serotypes are known. These include polioviruses 1 (Kitamura et al., 1981; Racaniello & Baltimore, 1981; Nomoto et al., 1982), 2 (Toyoda et al., 1984; La Monica
Fig. 2. Dendrogram showing the amino acid similarity in the capsid protein region (P1) between representatives of enteroviruses. The genetic clusters are indicated.

et al., 1986; Pevear et al., 1990) and 3 (Stanway et al., 1983, 1984; Toyoda et al., 1984, Hughes et al., 1986); coxsackieviruses A9 (Chang et al., 1989), A16 (Pöyry et al., 1994), A21 (Hughes et al., 1989), A24 (Supanaranond et al., 1992), B1 (Iizuka et al., 1987), B3 (Lindberg et al., 1987; Klump et al., 1990; Tracy et al., 1992; Chapman et al., 1994), B4 (Jenkins et al., 1987; Kang et al., 1994; Titchener et al., 1994) and B5 (Zhang et al., 1993); echoviruses 6 (V. F. Righthand and others,
unpublished), 9 (Zimmermann et al., 1995), 11 (Dahllund et al., 1995), 12 (Kraus et al., 1995), 22 (Hyypiä et al., 1992) and 23 (F. Ghazi and others, unpublished); enteroviruses 70 (Ryan et al., 1990) and 71 (Brown & Pallansch, 1995). Furthermore, partial sequence data are available from virtually all enterovirus serotypes (e.g. Pulli et al., 1995; Huttunen et al., 1996).

The sequences of three animal enterovirus serotypes have also been completed. These are BEV-1 (Earle et al., 1988), SVDV (Inoue et al., 1989, 1993; Seechurn et al., 1990) and PEV-9 (J. H. Peng and others, unpublished). Additionally, partial sequence data exist for BEV-2 (McNally et al., 1994), PEV-8 (J. H. Peng and others, unpublished) and PEV-10 (F. Lin and others, unpublished). The availability of this sequence information makes detailed genotypic analysis of the enterovirus genus possible.

Different parts of the genome have different functions and the rate of their evolution reflects this fact. The noncoding regions contain sequences that are recognized by the host cell ribosomal subunits as well as by factors playing a role in the replication of the genome. These specific motifs are highly conserved whereas, for instance, a sequence 100 nt long, preceding the initiation codon, shows extensive variation. On the other hand, the external capsid proteins are subjected to the pressure caused by host antibodies and exhibit notable variation in length and sequence of the loop structures connecting the β-sheets; this is evidently the reason why almost 70 different serotypes exist already among human enteroviruses. Nonstructural proteins have important functions in the life cycle of the virus and many of these polypeptides are highly conserved.

Overall comparison of the capsid proteins (P1 region) of different genera of picornaviruses (Fig. 1) reveals that representatives of enteroviruses group relatively tightly together. Rhinoviruses (HRVs) are the closest relatives of enteroviruses and the dendrogram does, in fact, show overlap between these genera in the P1 region. It is also clear from this analysis, and from comparisons of other regions of the genome either at the nucleotide or amino acid level that EV-22 (Hyypiä et al., 1992), together with EV-23 (Stanway et al., 1994; F. Ghazi and others, unpublished), represent an independent picornavirus group in primary structure terms. In addition, other biological properties, such as lack of the host-cell shut-off (Coller et al., 1990, 1991) and differences in the structural proteins (Stanway et al., 1994), support the idea of reclassification of these viruses outside the genus Enterovirus.

The dendrogram of P1 relationships (Fig. 2), together with those expressing the relationships between individual proteins (Pöyry et al., 1994; Dahllund et al., 1995) spanning the whole coding region, indicate the presence of four genetic clusters among human enteroviruses. CAV-2 and -16, together with enterovirus 71 (data not shown) are in one cluster (A); the second cluster (B) contains the four sequenced CBV serotypes (1, 3, 4 and 5), CAV-9, EV-11 and -12, and SVDV; the third cluster (C) consists of the three poliovirus serotypes together with CAV-21 and -24; enterovirus 70 alone forms a fourth subgroup. The difference between the enterovirus clusters exceeds that between enteroviruses and rhinoviruses.

Partial sequence analysis of the CAV VP4/2 protein coding region (Fig. 3, Table 1) suggests that, in addition to the virus serotypes shown in the dendrogram, certain CAVs belong to the same cluster as CAV-2 and -16 while, in addition to serotypes 21 and 24, some others are found in the poliovirus cluster (Pulli et al., 1995). All echovirus serotypes are grouped together with CBVs and CAV-9 when partial sequences of VP2 are analysed (Huttunen et al., 1996).

Grouping using the nonstructural protein region for comparisons follows essentially that found in the capsid region (Pöyry et al., 1996). 3D is an important protein to consider since it has been proposed that the classification of positive-strand RNA viruses could be based on amino acid identity in the RNA polymerases (Koonin & Dolja, 1993). Comparison of the 3D amino acid sequences shows that the members of groups containing the polioviruses and CAV-21 and -24 as well as the one containing CAV-9, CBV-1, -3, -4 and -5, and EV-11 and -12 each cluster very tightly together while CAV-16 and enterovirus 70 are relatively distantly related to these groups (not shown). Again, partial sequence analysis of CAV and EV serotypes in the 3D region supports this clustering among the other representatives of this virus group (Pulli et al., 1995; Huttunen et al., 1996).

Comparison of the available 5’UTR sequences leads to a grouping which is different from that obtained when protein sequences are compared. Only two clusters are observed: the first one contains polioviruses, CAV-21, CAV-24 and enterovirus 70, i.e. two of the genotypic subgroups based on protein identities, while CAV-9 and -16, CBV-1, -3, -4 and -5, and EV-

Table 1. Genetic classification of enteroviruses

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Members</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CAV serotypes and enterovirus 71</td>
</tr>
<tr>
<td>B</td>
<td>CAV-9, CBV serotypes 1–6, echoviruses, enterovirus 69, SVDV</td>
</tr>
<tr>
<td>C1</td>
<td>Poliovirus serotypes 1–3</td>
</tr>
<tr>
<td>C2</td>
<td>CAV serotypes</td>
</tr>
<tr>
<td>D</td>
<td>Enteroviruses 68 and 70</td>
</tr>
<tr>
<td>E</td>
<td>BEV serotypes 1 and 2</td>
</tr>
<tr>
<td>F</td>
<td>PEV serotype 8</td>
</tr>
<tr>
<td>G</td>
<td>PEV serotypes 9 and 10</td>
</tr>
</tbody>
</table>
11 and -12 are found in the other cluster, i.e. the members of the two other coding region groups of human enteroviruses (Pöyry et al., 1994; Dahllund et al., 1995). In the 3'UTR, three groups based on primary and predicted secondary structures can be formed. The length of this region in CAV-9, CBV-1, -3 -4 and -5, and in EV-11 and -12 is approximately 100 nt and can be considered to be made up of three secondary structure domains. Due to an apparent deletion directly after the termination codon, the length of the 3'UTR in polioviruses, CAV-21 and -24 is about 70 nt and the first predicted secondary structure domain is absent as it is in enterovirus 70. CAV-2 and -16 as well as enterovirus 71 represent an intermediate case (an approximately 80 nt 3'UTR with a truncated first secondary structure domain) in this respect (Hyypiä & Stanway, 1993; Pöyry et al., 1996). The biological significance of all the observed clustering is currently unknown.

The same criteria can also be applied to the non-human enteroviruses which have been sequenced. SVDV is closely related to CBV-5 (Zhang et al., 1993; Fig. 2) and clusters with this human pathogen in all analyses. In contrast, BEVs are distinct from the human enteroviruses and form a fifth coding region genotypic subgroup (cluster E) and a third 5'UTR subgroup (Table 2). However, in the 3'UTR they are members of the poliovirus-like cluster. Sequence data for PEV-8 (J. H. Peng and others, unpublished) are only available for part of the 5'UTR, the 3D polymerase and the 3'UTR; in the polymerase region, PEV-8 is the most distantly related member of the genus Enterovirus and has thus been assigned to a sixth cluster (F). The predicted secondary and tertiary structures of the PEV 3'UTR (82 nt) may resemble those of polioviruses; however, involvement of part of the protein-coding region would be required. In the 5'UTR, PEV-8 is most like, but may be distinct
from the CBV group. In the capsid-coding region and 3'UTR PEV-9 (J. H. Peng and others, unpublished) is most closely related to the BEVs; however, they are more distantly related in the nonstructural protein genes and thus PEV-9 has been assigned to a seventh cluster (G). The 5'UTR of PEV-9 is distinct and forms a fourth cluster. Analysis of part of the 5'UTR of PEV-10 shows it to be closely related to PEV-9 (F. Lin and others, unpublished).

### Diagnostic aspects

At present, diagnosis of enterovirus infections is carried out by isolation of the virus in cell culture followed by neutralization typing, or by demonstrating a virus-specific immune response in the host. Most of the enterovirus serotypes grow well in standard cell cultures, except that some CAVs can be isolated only in suckling mice. Because changes in cell morphology are not specific for any virus group, a confirmatory assay is needed for further typing of an isolated virus strain. Neutralization typing is therefore carried out by incubating the isolate with a panel of antisera pools and by subsequent evaluation of the inhibition of virus growth. As soon as more-specific receptor interactions have been thoroughly characterized, it may become possible to use cell lines, expressing a single enterovirus receptor on their surface, for virus isolation as has already been done in the case of poliovirus (Hovi & Stenvik, 1994).

The most specific method for serological diagnosis of enterovirus infections is based on differences in neutralization titres between serum samples from acute and convalescent phases of the disease. However, this requires the use of more than 60 virus strains and therefore is not in routine diagnostic use, although occasionally utilized in epidemiological studies. Cross-reactive antigens are more commonly used, allowing detection of enterovirus infection without more specific information of the serotype.

Progress in molecular virology and the introduction of new methods, especially PCR, have provided diagnostic laboratoires with new alternatives for enterovirus identification. Most of the PCR assays take advantage of the conserved sequences in the 5'UTR of the enterovirus genome (e.g. Hyyypiä et al., 1989), but approaches based on other regions (e.g. Olive et al., 1990) have also been described. The sensitivity of these assays is equal to or better than virus isolation and direct demonstration of viral RNA from clinical material has been reported in a number of studies. Therefore, these assays have the potential to replace virus isolation in routine diagnosis. One of the problems is the further typing of the viruses which could, in principle, be carried out by subsequent hybridization using specific probes or by sequence analysis of the amplicons. This, however, would require subgrouping based on genotypic rather than phenotypic and antigenic characteristics.

One of the most important criteria for such a classification would be a good correlation with both biological and clinical properties of the viruses. Of prime importance is the discrimination between polioviruses and non-polio enteroviruses. It is evident from the data presented above that discrimination cannot be done exclusively by analysing the genome regions used in standard PCR assays. However, more specific assays have been described (Yang et al., 1992) which could be used after the primary detection has appeared positive. When it comes to the other enteroviruses, clustering of CBVs, CAV-9 and EVs together would make up a large group with a highly similar pathogenic pattern. From a clinical point of view, it is usually sufficient to know that a virus belonging to this cluster has been identified. If necessary, further epidemiological analysis can be accomplished by partial sequencing of different regions of the genome. The same is true for the cluster containing CAV serotypes and enterovirus 71.

### A molecularly-based enterovirus subgrouping scheme

The biological and molecular data (Table 2) clearly indicate that the present subgroup classification of enteroviruses should be revised. The main reasons are the now redundant (for newly identified enteroviruses) subgrouping into polioviruses, CAVs, CBVs and EVs, and particularly the occurrence of CAVs in several of the clusters defined in sequence or receptor terms, rather than being in one coherent subgroup. Molecular and biological data clearly indicate that EV-22 and -23 form an independent picornavirus group and these viruses should be

### Table 2. Summary of the molecular, biological and pathogenic characteristics of the viruses in different enterovirus clusters

<table>
<thead>
<tr>
<th>Cluster</th>
<th>5'UTR</th>
<th>Coding region</th>
<th>Receptors</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>I</td>
<td>1</td>
<td>nk</td>
<td>CAV*</td>
</tr>
<tr>
<td>B</td>
<td>I</td>
<td>2</td>
<td>xβ1, xβ2, DAF, nk</td>
<td>CBV,† none</td>
</tr>
<tr>
<td>C1</td>
<td>II</td>
<td>3</td>
<td>PVR, CD44</td>
<td>Poliov+</td>
</tr>
<tr>
<td>C2</td>
<td>II</td>
<td>3</td>
<td>ICAM-1, nk</td>
<td>CAV</td>
</tr>
<tr>
<td>D</td>
<td>II</td>
<td>4</td>
<td>nk</td>
<td>nk</td>
</tr>
<tr>
<td>E</td>
<td>III</td>
<td>5</td>
<td>nk</td>
<td>CBV§</td>
</tr>
<tr>
<td>F</td>
<td>II?</td>
<td>6</td>
<td>nk</td>
<td>nk</td>
</tr>
<tr>
<td>G</td>
<td>IV</td>
<td>7</td>
<td>nk</td>
<td>nk</td>
</tr>
</tbody>
</table>

* Flaccid paralysis in newborn mice due to the involvement of muscle tissue.
† Spastic paralysis in newborn mice caused by the infection of the central nervous system.
‡ Paralysis in primates; some poliovirus strains can also infect mice.
§ Skeletal myonecrosis in mice similar to CBV infection (Adair et al., 1987).

nk, Not known.
reclassified as a new genus. Amino acid sequence analysis indicates that the rest of the human enteroviruses fall into one of four genetic subgroups, regardless of the protein being used for comparison, and suggests that this could form the basis of a rational classification scheme (Tables 1 and 2). Other molecular properties such as 5′UTR and 3′UTR sequences are also fully consistent with these groupings since, for instance, all members of group B (CBVs, CAV-9 and typical EVs) have a CBV-like 5′UTR and 3′UTR.

The non-human enteroviruses are distinct from their human counterparts and are thus easily differentiated; bovine and porcine enteroviruses are also easily distinguishable. The finding that PEV-8 and PEV-9 fall into different clusters is not surprising since they had been shown previously to belong to different PEV subgroups (Knowles et al., 1979).

One of the most surprising aspects of the molecular analyses is the lack of a distinct poliovirus group. These viruses have a relatively clearly defined pathogenicity yet consistently segregate with some of the CAVs in all the molecular analyses. In this case, it may be practical to use further criteria to allow identification of a subgroup containing only polioviruses. It is interesting that the non-poliovirus members of this genetic group apparently all utilize the ICAM-1 molecule (Pulli et al., 1989) in their entry into the host cells. In this case then, further differentiation can be made on this basis and our proposal is that the poliovirus-like genotypic cluster (C) should be divided into subgroups C1 and C2 (Fig. 3). Although there is a less compelling clinical case for dividing the other genetic clusters and sufficient information is at present lacking, receptor specificity might be a complementary criterion for the further division of these groups in the future.

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