Molecular typing and epidemiology of enteroviruses in Cyprus, 2003–2007

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Human enteroviruses (HEVs) are responsible for a wide spectrum of clinical diseases. Even though usually associated with non-specific febrile illness, they are the most common cause of viral meningitis and pose a serious public-health problem, especially during outbreaks. Rapid detection and identification of HEV serotypes in clinical specimens are important in appropriate patient management and epidemiological investigation. A 5 year study (2003–2007) of clinical specimens from patients with viral meningitis and/or symptoms of enteroviral infection was carried out in Cyprus to determine the underlying enteroviral aetiology. Reverse transcription, followed by a sequential PCR strategy targeting the 5' non-coding region and VP1 region, was used for typing the isolated enteroviruses. The serotype of each isolate was determined by BLAST search of the VP1 amplicon sequence against GenBank. Clinical specimens from a total of 146 patients were diagnosed as enterovirus-positive. Twenty-two different serotypes were identified. The main strains identified were echovirus 18 and echovirus 30, followed by coxsackievirus B5, echovirus 9, echovirus 6, coxsackievirus A10 and coxsackievirus B2. However, rapid changes in serotype frequency and diversity were observed over time. Serotype distribution corresponded essentially with observations reported from other European countries in the same period. The present report demonstrates the epidemiology of enteroviruses in Cyprus from 2003 to 2007.

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INTRODUCTION

The genus *Enterovirus* belongs to the family *Picornaviridae* and currently contains ten species, seven of which include viruses that commonly infect humans (<http://www.picornaviridae.com>). Human enterovirus (HEV) serotypes were recently reclassified into four groups (A–D), which include echovirus (E), coxsackievirus (CV) A and B, poliovirus (PV) and numbered enteroviruses (EVs) (Brown *et al.*, 2003; Pallansch & Oberste, 2003; Pallansch & Roos, 2001; Stanway *et al.*, 2005). HEVs are positive-sense, single-stranded RNA viruses that are transmitted faecal–orally and infect millions of people worldwide each year, with a seasonal peak in summer and autumn in temperate climates. Most infections are asymptomatic or result only in mild illness (such as non-specific febrile illness or mild upper respiratory symptoms). However, HEVs are a common aetiological agent in encephalitis, acute haemorrhagic conjunctivitis, undifferentiated rash, acute flaccid paralysis, myocarditis and neonatal sepsis-like diseases

(Pallansch & Roos, 2001). The most common of the potentially severe illnesses associated with EV infections, especially in paediatric patients, is aseptic meningitis, which often appears in the form of outbreaks. Non-polio enteroviruses (NPEV) are responsible for 85–95% of all cases of aseptic meningitis with an identified aetiological agent (Rotbart, 1995).

HEVs are distributed, within a given geographical area, with two major patterns of infection: endemic and epidemic (Pallansch & Oberste, 2003). This circulation of EVs and the periodic introduction of certain serotypes increase the possibility for the creation of new variants as a result of intratypic and intertypic recombination (McWilliam Leitch *et al.*, 2010; Simmonds & Welch, 2006). Recombination events have a significant role in the evolution and genetic variability of EVs (Santti *et al.*, 1999). Nevertheless, the genetic variability of EVs is caused mostly by the high mutation rate that generally characterizes RNA viruses, due to the lack of proofreading activity of the viral RNA-dependent RNA polymerase during genome replication (Drake & Holland, 1999).

Nowadays, there are numerous antigenically distinct serotypes because of these genetic-variation mechanisms.

Abbreviations: CSF, cerebrospinal fluid; CV, coxsackievirus; E, echovirus; EV, enterovirus; HEV, human enterovirus; NC, non-coding; NPEV, non-polio enterovirus; PV, poliovirus.

Therefore, the laboratory detection of EVs can no longer be based on serotyping procedures, due to the necessity for corresponding antibodies for recognition of the antigenic epitopes. In order to solve the problems of limited existence of type-specific antisera and cross-reactivity, cell-culture and molecular (such as PCR) assays are being used (Bolanaki *et al.*, 2008; Caro *et al.*, 2001; Casas *et al.*, 2001; Iturriza-Gómara *et al.*, 2006; Nix *et al.*, 2006; Oberste *et al.*, 2000, 2003). Molecular methods for the detection of HEVs rely on the fact that the genome of EVs has a highly conserved region in the 5' non-coding end, which contains the cloverleaf structure and the internal ribosome entry site. The most variable region is the VP1 region, which encodes serotype-specific neutralization epitopes. The hypervariable BC loop, within the N terminus of the VP1 protein, has an important role in reactivity with type-specific antibodies. These characteristics of the VP1 region explain why it is widely targeted for typing of EVs (Muir *et al.*, 1998; Norder *et al.*, 2001; Palacios *et al.*, 2002).

This study reports the molecular detection and typing of EVs isolated from clinical specimens from patients diagnosed with aseptic meningitis and other EV infections in Cyprus between 2003 and 2007.

METHODS

Patients and clinical samples. Between January 2003 and December 2007, our laboratory tested clinical specimens obtained from 246 hospitalized patients, from all Governmental Hospitals of Cyprus and from private clinics, with suspected viral meningitis or EV infection manifestations. The clinical samples were cerebrospinal fluid (CSF), pharyngeal swabs, blood samples and/or stool samples. Patients' ages ranged from newborn to 67 years, with >97% of patients being <15 years old. Children under 1 year accounted for 40% of patients of known age, followed by the groups of patients aged 1–5 years (32%) and 6–10 years (18%). In 2003 and from January to November 2004, clinical specimens were tested routinely in the laboratory by an in-house nested RT-PCR assay targeting a sequence in the highly conserved 5' NC region as described previously (Richter *et al.*, 2006). From December 2004, an in-house two-step real-time RT-PCR assay was employed for routine laboratory diagnosis of EVs. The molecular diagnostic analysis was positive for 146 of 246 patients. Ninety-one of 173 CSF samples (53%), 76 of 102 stool samples (75%) and five of five pharyngeal swabs (100%) were found to be EV-positive.

Extraction and real-time RT-PCR. Stool samples were treated as described by the WHO (2003). Viral RNA was extracted from 150 µl clinical specimen by using a QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. Real-time PCR for the detection of EV was performed as a two-step assay (separate reverse transcription followed by real-time PCR). For reverse transcription, 0.8 µl primer HEV 7 (25 µM) (Table 1), 0.8 µl 25 mM dNTP mix and 4.5 µl H₂O were added to 10 µl RNA. The mixture was heated to 95 °C for 5 min and subsequently snap-cooled on ice for 1 min. To each sample was added 3.9 µl of a reaction mixture containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 20 U RNase inhibitor and 30 U SuperScript III reverse transcriptase (Invitrogen). cDNA synthesis was performed for 30 min at 42 °C. cDNA product (7.5 µl) was added to 30 µl of a real-time PCR mixture containing 1× Universal TaqMan Mix (Applied Biosystems), 0.2 µM probe, 0.3 µM

Table 1. Oligonucleotide primers used for RT-PCR and the VP1 PCR

Primer	Sequence (5'→3')	Position*
Forward primers		
292B	ACIGCIGYIGARACNNG	2611–2627
292A	CAAGCIGCIGARACNNG	
292C	ACIGCIGTIGARACNNG	
Reverse primers		
222B	CCICCGGIGGIAYRTACAT	2969–2950
222A	GCICCGGIGGIACRWACAT	
222C	GCICCTGGIGGIAYRTACAT	
Reverse transcription		
HEV 7	ATTGTCAACCATAAGCAGCCA	594–575
HEV 6.1	CCCTGAATGCGGCTAATCC	449–467
EV probe	ACGGACACCCAAAGTAGTC GGTTCC	555–531

*Positions of primers are relative to the genome of PV type 1, Mahoney strain (GenBank accession no. V01148).

HEV 7 and 0.15 µM HEV 6.1 primers. All reactions were performed in duplicate. Incubation was carried out at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min in an ABI 7500 instrument (Applied Biosystems).

5' NC region nested RT-PCR. As described previously, our typing strategy is based on the sequencing of a large part of the 5' NC region and amplification of a part of the VP1 region (Richter *et al.*, 2006). Briefly, an approximately 399 bp fragment of the highly conserved 5' NC region was amplified by an in-house nested RT-PCR. Amplification involved 40 cycles of denaturation at 95 °C for 30 s, annealing at 42 °C for 45 s and elongation at 72 °C for 1 min. The approximately 399 bp products were visualized in a 1.5% agarose gel containing 1 µg ethidium bromide ml⁻¹.

Amplification of VP1-encoding sequences. The 222/292 primer set has a broad specificity and has been shown to amplify all known EV serotypes successfully, as well as to identify newly emerging serotypes (Oberste *et al.*, 2003). In order to do so, these primers contain several deoxyinosine residues and are highly degenerated, thereby limiting the sensitivity of amplification that can be achieved. It is possible to reduce the degree of degeneration by targeting groups of serotypes with high sequence similarity. Three different primer sets were developed (Table 1). Each of these primer sets is more sensitive and specific for a different group of EV serotypes. For reverse transcription, 2 µl random hexamers (15 µM; Invitrogen) and 8 µl of a 2.5 mM dNTP mix were added to 5 µl RNA. The mixture was heated to 95 °C for 5 min and subsequently snap-cooled on ice for 1 min. To each sample was added 5 µl of a reaction mixture containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 20 U RNase inhibitor and 30 U SuperScript III reverse transcriptase (Invitrogen). cDNA synthesis was performed at 25 °C for 10 min and at 42 °C for 45 min. Five microlitres of cDNA product was added to 35 µl of a PCR mixture containing a final concentration of 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.4 mM dNTPs, 0.5 µM of each primer (forward and reverse) and 2 U AmpliTaq Gold DNA polymerase (Applied Biosystems). Amplification and visualization by agarose gel electrophoresis were performed as described above; the amplicons were approximately 357 bp long.

Sequencing. Amplicons were purified by using a Montage DNA Gel Extraction kit (Millipore) and sequenced on a CEQ 8000 sequencer (Beckman Coulter) in the forward and reverse directions, using the respective PCR primers and a CEQ Dye Terminator Cycle Sequencing with Quick Start kit (Beckman Coulter). The sequencing results were used for a BLAST search against GenBank.

Phylogenetic analysis. Phylogenetic analyses were conducted by using MEGA4 (Tamura *et al.*, 2007). Phylogenetic trees were inferred by using the neighbour-joining method. Evolutionary distances were computed by using the *p*-distance method and are in units of the number of amino acid differences per site. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. All ambiguous positions were removed for each sequence pair. For reasons of clarity, interior branches representing distinct clusters were collapsed into elongated triangles, whose thickness is proportional to the number of taxa condensed and whose width is proportional to the maximum distance between taxa.

RESULTS

Over the 5 year period, EV RNA was detected in 228 clinical specimens obtained from 146 patients with suspected viral meningitis or manifestations of EV infection. One hundred and forty-six CSF, pharyngeal swab, blood and/or stool samples were analysed for epidemiological surveillance. In several cases, more than one specimen – e.g. CSF and stools – from a patient were used for typing. As the typing results were identical, for the purpose of simplicity, in the following results, the number of samples refers to the number of patients. A nested RT-PCR was employed to amplify part of the highly conserved 5' NC region. The sequences obtained were compared with those available in GenBank. Of the 146 samples, 141 were NPEVs belonging to species HEV-A and HEV-B, three were PVs and two corresponded to rhinoviruses. The three PVs were analysed further by sequencing of additional fragments in order to differentiate between the Sabin and wild-type strains (Richter *et al.*, 2008). All were identified to be Sabin-like PVs. One was type 3 Sabin PV and the other two were type 1 and type 2.

A phylogenetic tree was constructed from the aligned NPEV partial 5' NC region sequences and homologous sequences from GenBank, in order to guide the choice of the set of primer pairs for the VP1 RT-PCR (tree available on request). All of the VP1 sequences obtained were used as query sequences for comparison with those included in GenBank and each sample was assigned the serotype that gave the highest identity score, regarding the sequence similarity criteria defined by Oberste *et al.* (1999). A phylogenetic tree was constructed on the basis of an amino acid alignment of all VP1 fragments, including relevant reference strains from GenBank, by using the neighbour-joining method. In the VP1 tree (Fig. 1), clinical specimens of the same serotype clustered together with the respective GenBank reference sequences, further confirming the serotype identification and supported by high bootstrap values. All samples formed monophyletic clusters with the

Fig. 1. Phylogenetic tree based on partial VP1 sequences obtained from clinical specimens from patients with EV infection in Cyprus from 2003 to 2007 and reference sequences available in GenBank. All clinical isolates clustered with their respective reference strains with 99 % bootstrap values. Clinical samples are indicated in bold.

exception of CVB5, where two distinct lineages were observed. Unambiguous identification was achieved for 134 patients. A total of seven EV-positive specimens remained unidentified, either because the viral load was

too low, even after cell culture (unculturable serotype), or because the amount of sample was limited.

The distribution of the NPEV serotypes identified in Cyprus from 2003 to 2007 is shown in Table 2. Twenty-two different EV serotypes were identified. E18 and E30 were the most frequently isolated serotypes, followed by CVB5, E9, E6, CVA10 and CVB2. E7, E11, CVA14, CVA5 and CVB3 were detected sporadically. Serotypes CVB1 and CVA6 were each identified twice, while E13, E4, EV71, CVB4, CVA9, E2, CVA2 and CVA3 were each detected only once.

The number of EV-positive specimens varied throughout a single year and also between years. Every year, a low period of incidence was observed during winter and a high period from March to November, with a peak in summer and autumn. In 2007, a second peak was observed in December caused by E30. The overall NPEV-detection rate was highest in 2006 (39 %) and 2007 (32 %), with E18 (35 %) and E30 (52 %) being the most prevalent serotypes. 2004 was characterized by very low EV detection. In summer 2005, CVB5 (55 %) was the most frequently isolated serotype, followed by E9 (20 %) in autumn and winter. E9 was isolated until May 2006, when E6 appeared. In summer 2006, CVA10 was identified most frequently. E6 became predominant in September 2006, but its incidence declined rapidly in the following months as it was superseded by E18, which dominated until May 2007, when E30 became the most prevalent serotype. E30 peaked

in August 2007 and continued to dominate until December 2007, when it gave rise to a second bigger peak.

DISCUSSION

Worldwide, HEVs are responsible for a wide spectrum of clinical diseases. Even though usually associated with non-specific febrile illness, they can also cause severe infections and thus pose a serious public-health problem, especially during outbreaks. Rapid HEV detection and subsequent identification of the serotype in clinical cases is important for monitoring emergence and spread of more pathogenic variants, for epidemiological surveillance and for distinguishing between PVs and NPEVs (Antona *et al.*, 2007; Archimbaud *et al.*, 2009; Cabrerizo *et al.*, 2008; Mirand *et al.*, 2006). The genetic diversity of EVs makes it difficult to develop a molecular typing assay that is both sensitive and easy to perform (Leitch *et al.*, 2009; Nix *et al.*, 2006; Tavakoli *et al.*, 2008). However, many molecular methods have been developed for the typing of HEVs, most of them targeting the VP1 region. Data obtained by partial sequencing of the 5' NC region were used for distinguishing PVs from NPEVs (Hyypiä *et al.*, 1997; Thoelen *et al.*, 2004) and for guiding the choice of primers for the VP1 RT-PCR. Our typing approach, as described by Richter *et al.* (2006), became less laborious and turnaround time was reduced by employing the 222/292 primer set of Oberste *et al.* (2003). Furthermore, we were able to identify

Table 2. EV serotypes identified from 141 patients with EV infection in Cyprus from 2003 to 2007

	2003	2004	2005	2006	2007	Total
CVB1	0	1	0	0	1	2
CVB2	4	0	0	0	2	6
CVB3	0	0	1	2	0	3
CVB4	0	0	1	0	0	1
CVB5	1	0	11	0	0	12
E2	0	0	0	3	0	3
E4	1	0	0	0	0	1
E6	0	0	0	8	0	8
E7	0	0	1	4	0	5
E9	0	0	4	5	2	11
E11	0	0	0	1	4	5
E13	0	0	0	0	1	1
E18	3	0	0	20	8	31
E30	1	0	0	0	24	25
CVA2	1	0	0	0	0	1
CVA3	0	0	0	0	1	1
CVA5	0	0	0	2	1	3
CVA6	0	0	0	1	1	2
CVA9	0	0	1	0	0	1
CVA10	0	0	0	6	0	6
CVA14	3	2	0	0	0	5
EV71	0	0	1	0	0	1
Untyped	1	0	0	5	1	7
Total	15	3	20	57	46	141

a broader range of serotypes in clinical specimens, particularly rare or new serotypes. Because of the typically low viral loads in clinical samples (e.g. serum or CSF), an effort was made to reduce the degree of degeneration in primers 222 and 292, in order to increase the sensitivity of the assay. For this purpose, the 222/292 primer set was used as template for the development of three slightly modified primer sets (Table 1). Each of these primer sets is designed to match one specific HEV species (A, B or C) and offers a higher sensitivity with regard to this HEV species (data not shown). This approach proved especially useful for HEVs of group A, which are difficult to grow in cell culture and therefore typing must be performed using the original, usually low-concentration, sample. Employing this primer set to human HEV-B was also advantageous, as labour-intensive cell culturing could often be avoided.

In this study, EVs isolated from clinical specimens from 146 patients with EV-infection manifestations were analysed. The majority of the EV-positive isolates originated from hospitalized patients diagnosed with acute viral meningitis (63 %). In addition, 18 patients (12 %) suffered from gastroenteritis. For the rest of the patients, the reported main symptoms were fever, headache, rash and skin lesions (haemorrhagic exanthema; hand, foot and mouth disease). Fig. 2 shows the relationship between clinical symptoms and the serotypes of EV identified; in Fig. 3, the age distribution of the patients according to serotypes identified is shown. The majority of patients were <15 years of age. Serotypes E18 and E30, which caused outbreaks, were identified more frequently in children aged between 2 and 10 years old. Children under 1 year of age were more susceptible to sporadic infections by a wider range of different serotypes.

Although the samples originated from all governmental hospitals and private clinics in Cyprus, the two cities with

the largest populations made up >76 % of the isolates. The highest incidence of EV infections was observed in Nicosia (39 %), followed by Limassol (37 %). Nicosia is the capital of Cyprus and the biggest city on the mainland. Limassol is a coastal city and the second largest, welcoming the majority of tourists.

The most frequently identified NPEV serotypes were E18 (22.0 %) and E30 (17.7 %), followed by CVB5 (8.5 %), E9 (7.8 %), E6 (5.7 %), CVA10 (4.3 %) and CVB2 (4.3 %). It seems that these serotypes caused temporally separated outbreaks that succeeded each other. These findings are in accordance with other epidemiological studies, where predominant serotypes and ranking of individual EVs changed over time, as epidemics occurred (Antona *et al.*, 2007; Bahri *et al.*, 2005; CDC, 2003, 2006; Khetsuriani *et al.*, 2006a, b; Roth *et al.*, 2007; Trallero *et al.*, 2010; Tsao *et al.*, 2010). E18 emerged in Cyprus in 2003, causing sporadic cases before it became the predominant serotype in autumn of 2006 lasting until spring 2007. This trend was also reported in other EV-surveillance studies. While E18 was among the most frequent serotypes isolated in Europe and the USA after 2000 (Khetsuriani *et al.*, 2006b; Thoelen *et al.*, 2003), it seems that its incidence increased in 2005–2006 (Antona *et al.*, 2005; Mirand *et al.*, 2006; Roth *et al.*, 2007; Tavakoli *et al.*, 2008). The second most common serotype detected was E30. It was the causative serotype of the aseptic meningitis outbreak in Cyprus in epidemic years 2000–2001 (Richter *et al.*, 2006). As E30 has been prominent in meningitis outbreaks in France (2000 and 2005) (Antona *et al.*, 2005, 2007; Brunel *et al.*, 2008; Mirand *et al.*, 2006, 2008), Spain (1998, 2000, 2002, 2006 and 2007) (Cabrerizo *et al.*, 2008; Trallero *et al.*, 2010), Germany (2000–2001 and 2005–2006) (Roth *et al.*, 2007), Kosovo and Latvia (2006) (Eurosurveillance Weekly, 2006; Perevoscikovs *et al.*, 2006), Belgium and Austria (2000) (Ortner *et al.*, 2009; Thoelen *et al.*, 2003), its epidemiological status has been studied extensively (Bailly *et al.*, 2009; Kapusinszky *et al.*,

Fig. 2. Relationship between clinical symptoms and the serotype of EVs identified. The main clinical syndrome was viral meningitis. Serotypes E18, E30 and CVB5 were the most frequently detected in clinical specimens from patients with viral meningitis. Unspecific symptoms such as fever, headache, rash and diarrhoea have been included in 'other viral infection symptoms'.

Fig. 3. Age distribution of patients according to serotypes identified. The three most frequently detected serotypes (E18, E30 and CVB5), in clinical specimens from patients with viral meningitis, are shown separately.

2010; Lvque *et al.*, 2010; Lukashev *et al.*, 2008; McWilliam Leitch *et al.*, 2009, 2010; Mirand *et al.*, 2007). It has been suggested that E30 does not seem to be restricted geographically, but that a particular genotype circulates in different regions of the world simultaneously (Cabrerizo *et al.*, 2008). In addition, it has been reported that this serotype follows an epidemic mode of transmission, causing large outbreaks followed by periods of quiescence (Mirand *et al.*, 2007; Tavakoli *et al.*, 2008; Thoelen *et al.*, 2003). The incidences of E30 in Cyprus seem to follow this pattern. Whilst CVB5, E6 and E9 are among the most common serotypes reported in EV-surveillance studies (Bahri *et al.*, 2005; Cabrerizo *et al.*, 2008; CDC, 2003; Kapusinszky *et al.*, 2010; Khetsuriani *et al.*, 2006b; Mirand *et al.*, 2008; Tavakoli *et al.*, 2008; Trallero *et al.*, 2010), CVA10 is rarely reported. This difference may be attributed in part to the fact that CVA10 was isolated from throat swabs and stool specimens from patients with skin lesion diseases (haemorrhagic exanthema; hand, foot and mouth disease) or gastroenteritis, whereas acute meningitis is the main syndrome studied, especially in European countries. EV isolation from CSF collected from patients with neurological diseases has high significance. In contrast, the presence of EV in stools does not provide evidence of aetiology, as virus shedding may occur asymptotically and for a long period of time.

EVs demonstrate a marked seasonality in temperate climates, with a typical EV season occurring during June–October (Antona *et al.*, 2007; CDC, 2003). Our study showed a seasonal pattern of distribution, with the incidence of EV infections peaking in summer and autumn. In 2007, which can be characterized as an epidemic year, a second peak was observed in winter. This peak was caused by E30 and was higher than during the summer. Even though winter outbreaks of EVs are rarely reported (Antona *et al.*, 2007; Chambon *et al.*, 2001), in Cyprus they have been reported previously (Richter *et al.*, 2006) and are probably associated with persisting mild temperatures.

In general, the pattern of HEV serotypes circulating in Europe is similar to the pattern of serotypes observed in

Cyprus each year. HEVs have no known non-human host or reservoir, so their circulation is linked to host mobility (Morens & Pallansch, 1995). Even though Cyprus is an island, the large number of tourists (approx. 2 million) arriving each year in Cyprus, compared with the small population (approx. 800 000), is probably a major factor in the epidemiology of EVs in Cyprus.

No wild-type PV strain was detected during the 5 years of surveillance, although three Sabin-like PVs (vaccine strains) were detected. The last case of wild-type PV in Cyprus occurred in 1995 (Richter *et al.*, 2008). Since then, Cyprus belongs to the countries considered PV-free by the WHO.

In conclusion, this study covered five EV seasons, which, combined with the three seasons covered by Richter *et al.* (2006), comprise a total of 8 years surveillance of EV circulation in Cyprus. We observed a serotype distribution that corresponded largely to observations in other European countries during the same period. Molecular typing of EVs, along with the clinical severity associated with each serotype, will provide real-time warning for outbreaks and epidemics.

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