Serotyping and Genetic Characterization of Hand, Foot, and Mouth Disease (HFMD)-Associated Enteroviruses of No-EV71 and Non-CVA16 Circulating in Fujian, China, 2011–2015

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Background: Hand, foot, and mouth disease (HFMD) is a common contagious disease in infants; it is caused by multiple serotypes of human enterovirus (EV), which belongs to the enterovirus genus of the picornavirus family. According to sentinel surveillance, infection with EVs other than EV71 and CVA 16 have become increasingly common in recent years among HFMD patients, posing new challenges for HFMD control. This study aimed to explore the spectrum of serotypes in the other EVs (non-EV71 and non-CVA16) in Fujian province in southeastern China.

Material/Methods: We investigated 562 samples from EVs-infected HFMD patients with diagnosis confirmed by real-time RT-PCR with other EVs infection between 2011 and 2015. Nucleotide acid detection and the serotyping of the enteroviruses were also performed. The complete VP1 gene was amplified and sequenced. VP1-based phylogenetic analyses of CVA6, CVA10, CVA4, and CVA2 were also performed.

Results: Among the samples, 22 serotypes of the other EVs, which belong to 4 species of human enterovirus A-D, were identified. Of the 22 serotypes, CVA6 (57.8%) and CVA10 (21.0%) were most common, followed by CVA4 (6.8%) and CVA2 (2.7%). The other 18 serotypes accounted for 11.7% of samples, none of which exceeded 2%. Among 47 (8.4%) samples from patients with severe HFMD, 10 serotypes were identified and most samples belonged to CVA6 (20/47), followed by CVA10 (11/47). Entire VP1 comparison revealed that overall genetic identities were 96.7%, 96.3%, 94.4%, and 94.9% among strains within CVA6, CVA10, CVA4, and CVA2, respectively.

Conclusions: VP1-based phylogenetic analysis for the 4 predominant serotypes indicated various clades or sub-clades, which suggests the complex transmissions of other enteroviruses in Fujian.

MeSH Keywords: Enterovirus • Hand-Foot Syndrome • Phylogeography • Serotyping

Abbreviations: HFMD – hand, foot, and mouth disease; EV – enterovirus; CVA16 – coxsackievirus A 16; CVB – coxsackievirus B; ECV – echovirus; DEPC – diethyl pyrocarbonate; MEGA – molecular evolutionary genetics analysis

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Background

Hand, foot, and mouth disease (HFMD) is a common and contagious disease caused by multiple serotypes of enteroviruses (EVs). Of the HFMD-associated EVs, enterovirus 71 (EV71) is the leading causative pathogen, which is responsible for the severity and fatality of the disease [1,2]. Coxsackievirus A (CVA) 16 is another major serotype observed among HFMD patients with less severe clinical symptoms [3,4]. In addition to these 2 main serotypes, the other EVs (referred to as enteroviruses non-EV71 and non-CVA16 in this study), including CVA2, A4–6, A8–10, A12, A14, coxsackievirus B (CVB) 1–6, and echovirus (ECV) 3, 6, 9, 11, 24, and 25, have been frequently identified in HFMD patients in recent years in China [5–9] and other countries in Asia [10,11]. Of the other EVs, CVA6 and CVA10 were most frequently detected in HFMD patients in mainland China. In some Chinese provinces, CVA6 is even more prevalent than EV71 or CVA16, and has become the predominant serotype among HFMD patients [5,12]. Some of the serotypes had led to local outbreaks of HFMD [13]. The co-circulation of multiple serotypes of EVs has complicated the HFMD epidemic and poses great difficulties for disease control. Furthermore, some serotypes have been reported to be the causative pathogen of severe or fatal HFMD [14–16]. Most of the other EVs were clinically associated with mild symptoms, which also suggests the underestimated disease burden due to virus infection and mutation. A similar phenomenon was also observed in Fujian province in southeastern China.

Laboratorial surveillance of HFMD was implemented nationwide in 2008, at which time HFMD was categorized as a notifiable disease in mainland China. In the early stage of laboratory surveillance in Fujian, most laboratory surveillance focused on 2 main pathogens – EV71 and CVA16 – which cause severe and highly fatal HFMD. However, the EVs of non-EV71 and non-CVA16 were rarely serotyped because they were previously infrequently detected. As the proportions of other EVs have been increasing in recent years, more attention has shifted to the other EVs, especially for a vaccine against EV71, which was well developed and closer to commercialization.

In order to control HFMD, it is necessary to epidemiologically and etiologically characterize the other EVs. In this study, we conducted an investigation to serotype and explore the genetic characterization of the major EVs of non-EV71 and non-CVA16 associated with HFMD reported in Fujian province during 2011–2015.

Material and Methods

Sample collection

Considering the temporal and spatial distribution, a total of 562 throat or rectal swabs from HFMD patients with other EVs infection were involved in this study. Of the samples, 47 were from severe, non-fatal HFMD cases and the rest were from mild cases. During 2011–2015, 17 229 out of 24 589 samples were collected through sentinel surveillance hospitals in Fujian during 2011–2015. The detection for the positive nucleotide of the other EVs was performed using real-time RT-PCR tests. The demographic information and clinical data of patients were also collected through sentinel hospitals. The diagnosis of HFMD and the categories of mild, severe, and fatal case were defined according to the recommendation of the Chinese CDC [17].

This study was approved by the Ethics Review Committee of the Fujian province CDC. All information collected from patients, including demographic data, clinical records, and laboratorial findings, was approved by the patients, and all patients gave consent for this study. We promised the patients that the information would be kept anonymous for privacy protection. The patients who participated in this retrospective analysis had received regular medical examinations before the start of this study. Therefore, written content from patients was waived.

Nucleotide acid detection and serotyping of enteroviruses

RNAs were extracted from 140 μL supernatants of specimens by Viral RNA mini kit (Qiagen, USA) and eluted into 50 μL diethyl pyrocarbonate (DEPC) treated de-ionized water. We performed conventional RT-PCR with universal primer PE1/PE2, as recommended by the Chinese CDC [17], for re-confirmation of the other EVs infections of the selected patients. For serotyping the enteroviruses, 50 μL reaction mixture containing 5 μL viral RNA, 400 μM dNTP, and 0.4 μM primer of 292/222 [18] were prepared and amplified as follows: 45°C for 45 min; 95°C for 2 min; 40 cycles of 94°C for 30 s, 45°C for 30 s, 68°C for 50 s, and an extra holding at 68°C for 10 min. The amplified products were gel-purified and bi-directionally sequenced by using BigDye terminator cycle sequencing reagent v3.1 (ABI, USA). All sequences obtained in this study were subjected to the online blast tool (http://www.rivm.nl/mpf/enterovirus/typing tool) and the serotyping results of enteroviruses underwent automatic feedback.

Complete VP1 amplification and sequencing

To obtain entire VP1 sequences of other EVs, fragmented amplification and sequencing strategy were used and a set of primers [19] specific for human enterovirus amplification were used in this study. By using the One-step RT-PCR kit (Qiagen, USA), 50 μL reaction mixture containing 5 μL viral RNA, 400 μM dNTP, and 0.4 μM of each primer of both orientations was prepared and was amplified as follows: 50°C for 30 min, 95°C for 15 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, 70°C for 60 s, and an extra holding at 72°C for 10 min. The amplified
products were gel extracted and the purified products were bi-directionally sequenced by using BigDye terminator cycle sequencing reagent v3.1 (ABI, USA). Fragmented sequences were spliced by SeqMan program (DNAstar software, ver 7.1.0) to assemble the entire VP1 sequences.

**VP1-based phylogenetic analyses of CVA6, CVA10, CVA4, and CVA2**

In this study, phylogenetic analyses of 4 predominant serotypes of CVA6, CVA10, CVA4, and CVA2 were performed. Entire VP1 sequences of identical serotypes were aligned with the reference sequences retrieved from the GenBank by using the Clustal W program packaged in Molecular Evolutionary Genetics Analysis software (MEGA, ver 6.0). Phylogenetic trees based on entire VP1 genes were built using the neighbor-joining method in MEGA 6.0 software. Validation of reconstructed evolution trees were supported statistically by using the bootstrap with 1000 replicates.

**Results**

**Laboratory surveillance profile in Fujian during 2011–2015**

According to laboratory surveillance of EVs in Fujian, EV71 was the most commonly detected and CVA16 was the second most commonly detected from HFMD patients before 2011 from HFMD sentinel hospitals. In 2011, other EVs outnumbered EV71 and CVA16 for the first time. During 2011–2015, 17 229 out of 24 589 samples collected from HFMD sentinel hospitals were EV-positive according to the real-time RT-PCR analysis. Of the positive samples, EV71, CVA16, and other EVs accounted for 32.0% (5506/17229), 18.9% (3262/17229), and 49.1% (8461/17229), respectively. Monthly distribution of serotypes with overall EV positive rate of 69.3% (51.9% [193/372] to 83.9% [527/628]) during 2011–2015, which shows that other EVs predominated periodically in the periods of September 2012 to December 2013, August to December 2014, and July to December 2015, but during the rest of this time, EV71 (January–August 2012 and January–June 2015) and CVA16 (January–July 2014) alternately predominated (Figure 1). The 562 samples included in this study account for 6.6% (562/8461) of other EVs-positive samples collected in Fujian during 2011–2015.

**Serotyping of other EVs**

Among these 562 samples, 22 serotypes of other EVs belonging to 4 species were identified by partial VP1 sequencing. There were 12, 17, 5, 8, and 5 serotypes were identified in 2011, 2012, 2013, 2014, and 2015, respectively. The most prevalent serotype was CVA6 (57.8%, 325/562), followed by CVA10 (21.0%, 118/562), CVA4 (6.8%, 38/562), and CVA2...
The remaining 18 serotypes account for 11.7% (66/562) of the samples (Table 1), no one of which exceeds 2%. In view of species, most of other EVs of 7 serotypes (92.2%, 518/562) belonged to the species enterovirus A, and 13 serotypes belonged to the species enterovirus B, which accounts for 7.5% (42/562). It is noteworthy that 2 infrequent serotypes of CVA21 and EV68, which belong to the enterovirus species C and D, respectively, were identified from a single specimen each in this study.

### Serotypes associated with severe HFMD

In this study, 47 out of 562 samples (8.4%) were collected from patients with severe, non-fatal HFMD. Of these 47 samples, 10 serotypes were identified. The most frequently detected was CVA6 (20/47), followed by CVA10 (11/47), CVA4 (4/47), CVA2 (3/47), CVA5 (3/47), ECV6 (2/47), CVA9 (1/47), CVB3 (1/47), CVB5 (1/47), and ECV25 (1/47).

### Phylogenetic analyses of CVA6

We conducted entire VP1 sequence-based phylogenetic analyses of 4 predominant serotypes of CVA6, CVA10, CVA4, and CVA2. The detailed genetic characteristics are described below.

Entire VP1 genes of 41 CVA6 sequenced in this study shared the overall nucleotide identity of 96.7% (87.2% to 100%). A VP1-based evolutional dendrogram of CVA6 separated the viruses into 7 clades – A to G – and clade G could also be further divided into G1 to G4 sub-clades according to the phylogenetic relations (Figure 2). Most of the strains included in this study belong to G3 and G4 sub-clades, except for 2 strains (ZZ156/FJ/CHN/2011 and QZ073/FJ/CHN/2011) in 2011, which

### Table 1. Serotyping of other enteroviruses from patients with HFMD in Fujian, 2011–2015.

| Species     | Serotype | 2011 | 2012 | 2013 | 2014 | 2015 | Total | Proportion (%) |
|-------------|----------|------|------|------|------|------|-------|----------------|
| **Enterovirus A** |          |      |      |      |      |      |       |                |
| CVA2        |          | 3    | 3    | 4    | 0    | 5    | 15    | 2.67           |
| CVA4        |          | 0    | 31   | 0    | 2    | 5    | 38    | 6.76           |
| CVA5        |          | 1    | 2    | 6    | 1    | 0    | 10    | 1.78           |
| CVA6        |          | 23   | 103  | 56   | 17   | 126  | 325   | 57.83          |
| CVA8        |          | 1    | 3    | 0    | 0    | 3    | 7     | 1.25           |
| CVA10       |          | 32   | 38   | 26   | 7    | 15   | 118   | 21.00          |
| CVA12       |          | 4    | 1    | 0    | 0    | 5    | 10    | 0.98           |
| **Enterovirus B** |          |      |      |      |      |      |       |                |
| CVA9        |          | 3    | 2    | 0    | 1    | 0    | 6     | 1.07           |
| CVB1        |          | 3    | 0    | 0    | 0    | 3    | 6     | 0.53           |
| CVB2        |          | 1    | 0    | 0    | 0    | 1    | 2     | 0.18           |
| CVB3        |          | 1    | 7    | 0    | 0    | 7    | 15    | 2.53           |
| CVB4        |          | 0    | 5    | 0    | 0    | 5    | 10    | 1.78           |
| CVB5        |          | 3    | 1    | 0    | 2    | 0    | 6     | 1.07           |
| ECV3        |          | 0    | 1    | 0    | 0    | 1    | 2     | 0.36           |
| ECV6        |          | 1    | 2    | 0    | 1    | 0    | 4     | 0.71           |
| ECV7        |          | 0    | 1    | 0    | 0    | 1    | 2     | 0.36           |
| ECV9        |          | 0    | 1    | 0    | 0    | 1    | 2     | 0.36           |
| ECV16       |          | 0    | 2    | 0    | 0    | 2    | 4     | 0.71           |
| ECV25       |          | 0    | 2    | 0    | 0    | 2    | 4     | 0.71           |
| ECV30       |          | 2    | 1    | 0    | 0    | 3    | 6     | 1.07           |
| **Enterovirus C** |      |      |      |      |      |      |       |                |
| CVA21       |          | 0    | 0    | 1    | 0    | 0    | 1     | 0.18           |
| **Enterovirus D** |  |      |      |      |      |      |       |                |
| EV68        |          | 0    | 0    | 1    | 0    | 1    | 3     | 0.53           |

(2.7%, 15/562). The remaining 18 serotypes account for 11.7% (66/562) of the samples (Table 1), no one of which exceeded 2%. In view of species, most of other EVs of 7 serotypes (92.2%, 518/562) belonged to the species enterovirus A, and 13 serotypes belonged to the species enterovirus B, which accounts for 7.5% (42/562). It is noteworthy that 2 infrequent serotypes of CVA21 and EV68, which belong to the enterovirus species C and D, respectively, were identified from a single specimen each in this study.

(3/47), CVA5 (3/47), ECV6 (2/47), CVA9 (1/47), CVB3 (1/47), CVB5 (1/47), and ECV25 (1/47).
Figure 2. Entire VP1-based evolutional dendrogram of CVA6 circulating in Fujian, 2011–2015. CVA6 identified in 2011, 2012, 2013, 2014, and 2015 and sequenced in this study are marked by solid circles in red, blue, green, purple, and brown, respectively, before the name of viral strains. Bootstrap values more than 75% are indicated. The names of countries are abbreviated as CHN, China; ESP, Spain; FIN, Finland; FRA, France; IND, India; JPN, Japan; and USA, United States. The provinces in China are abbreviated as FJ, Fujian; GD, Guangdong; HN, Henan; SH, Shanghai; TJ, Tianjin; and YN, Yunnan.
belong to clade E. In contrast to clade E, the viruses of sub-clade G3 and G4 were predominant in Fujian in recent years. In particular, sub-clade G4 tended to become the main branch responsible for the CVA6 epidemic in Fujian because it comprised viruses that emerged 1–2 years later than the viruses of sub-clade G3. However, the possibility of co-circulation of 2 sub-clades is not excluded because 2 viruses (NP141/FJ/CHN/2015 and QZ099/FJ/CHN/2015) found in 2015 still remained on G3.

Phylogenetic analyses of CVA10

Fifty-two VP1 sequences of CVA10 obtained in this study shared overall nucleotide identity of 96.3% (86.6% to 100%). A VP1-based evolutionary dendrogram of CVA10 revealed that the viruses could be phylogenetically divided into clades A–D (Figure 3). Clade A comprised the prototype of CVA10 (Kowalik/USA/1949) only and the clade B comprised the strains found in France in 2010. Clade C consists of the strains that circulated early in 2004–2009 in China. Together with the viral strains found in other provinces in the same period, all CVA10 from Fujian during 2011–2015 belonged to clade D, without obvious spatiotemporal cluster, as the viruses were dispersed to nearly all twigs of the clade, indicating that the genetically similar CVA10 would be likely to circulate continuously in Fujian.

Phylogenetic analyses of CVA4

Only CVA4 samples collected in 2012 were VP1 sequenced and the samples in 2014–2015 were not sequenced due to failed VP1 amplification and sequencing in this study. Twenty-two VP1 sequences of CVA4 shared overall nucleotide identity of 94.4% (89.8–100%). A VP1-based evolutionary dendrogram of CVA4 separated the viruses into clades A–E, and clade E could be further divided into sub-clade E1 and E2 (Figure 4). Sub-clade E1 comprised 98401/SD/CHN/1998 only, which represents the CVA4 that circulated in the early stage of the epidemic in China. In contrast, sub-clade E2 consisted of all the contemporary CVA4 circulating in China, regardless of the viruses from Fujian or other provinces. Accordingly, CVA4, which belongs to sub-clade E2, would be likely to circulate continuously in Fujian or even in all of China.

Phylogenetic analyses of CVA2

Seven CVA2 samples collected from 2011 to 2013 were VP1 sequenced in this study. The overall nucleotide identity of CVA2 strains was 94.9% (92.3–98.4%). A VP1-based evolutionary dendrogram of CVA2 separated at the viruses phylogenetically into clades A–D, and clade D could be further divided into sub-clade D1 and D2 (Figure 5). Of the 7 CVA2s from Fujian, 4 belong to sub-clade D1 and 3 belong to D2. In contrast to D1, the sub-clade D2 comprised strains that appeared later, suggesting that the viruses of D2 are likely to become the main lineage among HFMD patients in Fujian.

Discussion

Infection with multiple serotypes of EVs, in particular the members of enterovirus A species, can cause HFMD. In the past, EV71 and CVA16 had drawn much attention due to their larger proportion among viral infections and their more severe clinical outcomes. Laboratorial surveillance demonstrated that other EVs have been increasingly detected among patients with HFMD in recent years. In addition, the predominance of other EVs over the 2 main pathogens of EV71 and CV16 was observed since 2011. For accurate description of the etiological characteristics of HFMD, we conducted serotyping of other EVs during 2011–2015 in Fujian; 22 serotypes belonging to 4 enterovirus species were identified from patients with HFMD. In addition, we sequenced the entire VP1 of partial viruses of 4 predominant serotypes of CVA6, 10, 4, and 2. Comparison of VP1 sequences between the viruses within identical serotypes revealed different genetic homogeneities. Due to lack of criteria for genetic categories for the 4 serotypes selected in this study, we divided the viruses into clades or sub-clades according to the phylogenetic relationships between the viruses of identical serotypes. We made a rough prediction of predominant clades or sub-clades of viruses that would circulate continuously in the future, based on the phylogenetic relationships between and temporal sequences of emergence of viruses.

Of the 22 serotypes identified in this study, CVA6 is the most prevalent and accounted for 57.8% of HFMD patients with other EVs infections during 2011–2015 in Fujian. Epidemics of CVA6 have occurred in countries in Asia, Europe, and America [20] since the first outbreak in Finland in 2008 [21], and is now widespread globally. In several regions in China, CVA6 has become even more common than EV71 and CVA16 in recent years, which indicates the changing nature of the HFMD epidemic in China [5,12]. CVA10 is another important serotype and accounted for 21.0% of other EVs in Fujian during 2011–2015. CVA10 had previously been reported as a cause of herpangina in Japan in the early 2000s [22], and the circulation of CVA10, together with CVA6, was confirmed to be responsible for the HFMD outbreak in Finland in 2008 [23]. In recent years, several regions, including Shandong, Ningxia, Guangdong, and Hubei, have reported the increasing occurrence of CVA10 infection, which makes it a common cause of HFMD in China [6,24–26]. For instance, serotyping by GeXP assay revealed that 18.25% of HFMD patients in Jinan in Shandong province were the cause of CVA10 infection [6]. Clinically, infection with CVA6 was associated with atypical HFMD with delayed onychomadesis [21]. Some HFMD patients with CVA6 infection could potentially develop complications with central nervous system involvement [14]. For CVA10, the severe clinical outcome due to viral infection could reach 36.6% according to a previous investigation [9]. We estimated that infection with CVA6 and CVA10 was responsible for about 40% of HFMD cases (49.1%
Figure 3. Evolutional dendrogram of CVA10 circulating in Fujian, 2011–2015. CVA10 identified in 2011, 2012, 2013, 2014, and 2015 and sequenced in this study are indicated solid squares in red, blue, green, purple, and brown, respectively, before the name of viral strains. Bootstrap values more than 75% are indicated. The names of countries are abbreviated as CHN, China; ESP, Spain; FRA, France; and USA, United States. The provinces in China are abbreviated as CQ, Chongqing; FJ, Fujian; HN, Henan; HeB, Hebei; HLJ, Heilongjiang; HuN, Hunan; NX, Ningxia; SD, Shandong; SZ, Shenzhen; YN, Yunnan; and ZJ, Zhejiang.
Figure 4. Evolutionary dendrogram of CVA4 circulating in Fujian, 2012. CVA4 identified in 2012 and sequenced in this study are marked by diamonds in blue. The names of countries are abbreviated as CHN, China; ESP, Spain; FRA, France; and USA, United States. The provinces in China are abbreviated as BJ, Beijing; CQ, Chongqing; FJ, Fujian; HN, Henan; HeB, Hebei; HuN, Hunan; HLJ, Heilongjiang; NX, Ningxia; SD, Shandong; SZ, Shenzhen; YN, Yunnan and ZJ, Zhejiang.
CVA4 and CVA2 were not detected every year during 2011–2015 in Fujian. Nonetheless, the proportion of each serotype exceeded 2.0% (6.8% of CVA4 and 2.7% of CVA2). Considering the 49.1% of other EVs-positive rate, it is estimated that each of them could exceed 1.0% among all EV-associated HFMD cases in Fujian. Infection with CVA4 frequently involved herpangina [16,27] and was occasionally involved in local outbreaks of febrile disease [13]. Similarly, infection of CVA2 was usually...
related to the herpangina, which manifests as high-grade fever, oral ulcers, and, less commonly, as skin rash [28]. In mainland China, CVA4 and CVA2 were also frequently detected serotypes in recent years [5–7]. Due to the different clinical manifestations of typical HFMD, particularly the unapparent skin rash, circulation of the 2 serotypes might lead to misdiagnosis and subsequently delayed clinical therapy.

Compared to the 4 serotypes of CVA6, 10, 4, and 2 mentioned above, the other 18 serotypes were detected infrequently (less than 2.0% each) in Fujian. It was noteworthy that, in addition to the serotypes belonging to enterovirus A and B species, 2 serotypes of CVA21 of Enterovirus C and EV68 of Enterovirus D were detected from a single sample each. CVA21 and EV68 were frequently related to respiratory tract infection [29–31] and rarely detected in patients with HFMD. Infection with EV68 can lead to outbreaks of acute respiratory disease [32] and occasionally causes central nervous system symptoms [15,33]. Widespread EV68 infection in recent years has raised global public health concerns [34,35]. A recent investigation revealed that CVA21 existed in environmental sewage and was detected in a few patients with acute flaccid paralysis [36]. The epidemiological significance of finding these 2 serotypes of EVs among HFMD patients remains unclear, and the environmental distribution and potential neurological pathogenicity of CVA21 and EV68 underscore the importance of further research on this public health problem.

Conclusions

EV71 and CVA16 are the 2 main pathogens responsible for the pandemic of HFMD in China since 2008, and the former was more often associated with the severity and high fatality of the disease [6,14,37,38]. At the end of 2015, a cost-effective prophylactic vaccine (inactivated) against EV71 was licensed to be marketed in mainland China [37,38]. Additionally, multiple forms of vaccines and therapy against CVA16 are also being developed [4,39–41]. It is predicated that mass immunization with an EV71 vaccine would dramatically decrease the severity and fatality of HFMD. However, co-circulation of other EVs of multiple serotypes still remains an issue for HFMD control. Detection, serotyping, and genetic characterization of other co-circulating EVs are conducive to understanding the epidemic of other EVs and determining the next priority serotype for vaccine development, as well contributing to development of strategies for HFMD control.

Statement

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Conflict of interests

None.

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