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Direct Detection of Enterovirus 71 (EV71) in Clinical Specimens from a Hand, Foot, and Mouth Disease Outbreak in Singapore by Reverse Transcription-PCR with Universal Enterovirus and EV71-Specific Primers

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A recent outbreak of hand, foot, and mouth disease in Singapore in 2000 affected several thousand children and resulted in four deaths. The aim of this study was to determine the applicability of reverse transcription-PCR (RT-PCR) with universal pan-enterovirus primers and enterovirus 71 (EV71) type-specific primers for the direct detection of enteroviruses in clinical specimens derived from this outbreak. With the universal primers, EV71 RNA sequences were successfully detected by RT-PCR and direct sequencing in 71% of positive specimens. Three pairs of EV71 type-specific primers were evaluated for rapid detection of EV71 directly from clinical specimens and cell culture isolates. By using a seminested RT-PCR strategy, specific identification of EV71 sequences directly in clinical specimens was achieved, with a detection rate of 53%. In contrast, cell culture could isolate EV71 in only 20% of positive specimens. EV71 was detected directly from brain, heart, and lung specimens of two deceased siblings. Although more than one type of enterovirus was identified in clinical specimens from this outbreak, 90% of the enteroviruses were confirmed as EV71. The data demonstrate the clinical applicability of pan-enterovirus and seminested RT-PCR for the detection of EV71 RNA directly from clinical specimens in an outbreak situation.

Hand, foot, and mouth disease (HFMD) is of serious concern in the Asia-Pacific region as a result of two major epidemics with high fatalities in Malaysia and Taiwan in recent years (6, 7, 11). The recent HFMD outbreak in Singapore in October 2000 affected several thousand children and resulted in four deaths (1). In Singapore, HFMD is endemic and the disease manifests mainly as febrile upper respiratory illness and herpangina. There have been reports of some children with acute flaccid paralysis who fully recovered (12). The classical diagnostic method for enteroviruses is propagation by cell culture followed by neutralization with specific antisera to confirm the serotype. Cell culture from clinical specimens is considered the "gold standard," but the procedure is time-consuming and may take a few weeks. Furthermore, some enteroviruses replicate poorly in cell cultures, require several passages, or may be untypeable (9).

In view of its high sensitivity and specificity, reverse transcription-PCR (RT-PCR) is beneficial for the rapid detection of enterovirus 71 (EV71) in outbreak situations. The rapid establishment of enteroviral etiology would eliminate the unnecessary use of antibiotics and lead to better patient management. Early diagnosis can help to limit the spread of the disease in the community during an outbreak. The aim of this study was to identify the etiological agents involved in the HFMD outbreak. We investigated the applicability of RT-PCR by using universal pan-enterovirus and type-specific primers for the direct detection of EV71 in clinical specimens. Direct sequencing of RT-PCR products amplified from the 5′ untranslated region (5′ UTR) by using universal primers (10, 16) was compared with a newly developed seminested RT-PCR assay based on specific primers targeting the VP1 region of EV71.

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MATERIALS AND METHODS

Clinical specimens. A total of 51 clinical specimens obtained from 22 pediatric patients suffering from HFMD in Singapore during the period from September to October 2000 were included in this study (Table 1). Most of the specimens were collected from children hospitalized with HFMD and from autopsied cases. The samples included stool, rectal swabs, vesicular swabs, mouth ulcers, nasopharyngeal aspirates, throat swabs, tracheal swabs, and brain, heart, lung, intestinal, lymph node, spleen, and tonsillar tissues. The specimens were directly subjected to RNA extraction followed by RT-PCR. In addition, specimens (minimum volume of 0.2 ml in virus transport medium) were also inoculated into RD and HeLa cells, and the virus isolates were typed by the microneutralization method with the Lim Benyesh-Melnick antiserum pool. The identification of EV71 was further confirmed with pooled EV71-specific antisera.

RNA extraction and RT-PCR. RNA was extracted directly from clinical specimens that were as low as 100 μl in volume. The specimens were lysed with guanidine isothiocyanate, and RNA was extracted by the silica adsorption method with minor modifications (5). The RNA pellet was reconstituted in 10 μl of sterile distilled water containing RNase inhibitor (Promega, Madison, Wis.) and stored at −80°C prior to use. The yield of RNA was determined by optical density measurements at 260 nm. Total RNA (2 to 5 μg) was mixed with 0.3 μg of random hexamer oligonucleotides (Life Technologies, Gaithersburg, Md.) and incubated at 70°C for 10 min. To this reaction mixture was added the first-strand buffer, 0.01 M dithiothreitol, 0.5 mM deoxynucleoside triphosphate mix, and 10 U of reverse transcriptase (Life Technologies) in a final volume of 20 μl. The RT reaction mixture was incubated at 42°C for 50 min and heat inactivated at 70°C for 15 min. The cDNA was stored at −80°C and used as a

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TABLE 1. Comparative detection of enteroviruses in clinical specimens by cell culture and two RT-PCR assays

Patient no.	Specimen identification no.	Source	Status of patient	Result by: Cell culture and neutralization test	Result by: RT-PCR with universal primers	Result by: Seminested PCR with EV71 specific primers	GenBank accession no.
1	16/555O/SIN0/01509	Throat swab	Alive	–[a]	–	–	
2	20/5589/SIN/001809	Stool	Alive	–	CA16	ND[b]	AF314004
	23/5602/SIN/001809	Stool	Alive	–	CA16	ND	
3	21/5593/SIN/001809	NPA[c]	Alive	–	–	–	
4	22/5598/SIN/001909	NPA	Alive	–	EV71	EV71	AF314003
	25/5628/SIN/002009	Mouth	Alive	–	–	–	
	30/5666/SIN/002209	Stool	Alive	EV71	EV71	–	AF352027
5	26/5656/SIN/002209	Vesicle swab	Alive	–	CVB4	ND	AF314005
6	27/5657/SIN/002209	Throat swab	Alive	–	–	–	
7	28/5658/SIN/002209	Throat swab	Alive	–	–	EV71	
8	29/5660/SIN/002209	Throat swab	Alive	–	–	–	
9	12/5555/SIN/001509	Vesicle swab	Alive	–	–	–	
	13/5556/SIN/001509	Mouth swab	Alive	–	–	EV71	
	11/5554/SIN/001509	Throat swab	Alive	–	–	EV71	
10	14/5557/SIN/001509	Oral	Alive	–	–	–	
	9/5536/SIN/001409	Stool	Alive	–	EV71	EV71	AF314007
11	18/5575/SIN/001809	Oral ulcer	Alive	–	–	–	
12	19/5576/SIN001809	Vesicle swab	Alive	–	–	–	
13	7/5506/SIN/001309	Vesicle swab	Alive	–	EV71	EV71	AF314006
14	8/5520/SIN/001309	Mouth ulcers	Alive	–	–	EV71	
15	10/5542/SIN/001509	Oral swab	Alive	–	–	EV71	
16	15/5545/SIN/001409	Mouth swab	Alive	–	–	–	
17	6/5505/SIN/001309	Mouth ulcers	Alive	–	–	EV71	
18	RS/SIN/001108	Rectal swab	Alive	–	EV71	EV71	
	TS/SIN/001002	Throat swab	Alive	–	EV71	–	AY027864
19	1/5466/SIN/001109	Lung	Dead	–	–	–	
	2/5467/SIN/001109	Heart	Dead	–	–	–	
	3/5468/SIN/001109	Intestine	Dead	–	–	–	
	4/5469/SIN/001109	Brain	Dead	–	–	–	
20	5546/SIN/000009	Rectal swab	Dead	–	EV71	–	AF314008
21	5786/SIN/000009	Rectal swab	Dead	–	–	–	
	5811/SIN/000009	Foot swab	Dead	–	–	EV71	
	5813/SIN/000009	Saliva	Dead	–	EV71	–	
	5814/SIN/000009	Rectal swab	Dead	EV71	EV71	–	AF316321
	5861/SIN/000009	Lung	Dead	–	EV71	–	
	5862/SIN/000009	Heart	Dead	–	EV71	–	
	5863/SIN/000009	Brain	Dead	–	EV71	–	
	5864/SIN/000009	Tracheal swab	Dead	EV71	EV71	EV71	
	5866/SIN/000009	Spleen	Dead	–	EV71	–	
	5867/SIN/000009	Oral swab	Dead	–	EV71	–	
	5868/SIN/000009	Intestine-stool	Dead	EV71	EV71	–	
	5869/SIN/000009	Tonsils	Dead	EV71	EV71	EV71	
22	5806/SIN/000009	Foot swab	Dead	–	EV71	–	
	5808/SIN/000009	Saliva	Dead	–	EV71	EV71	
	5809/SIN/000009	Rectal swab	Dead	EV71	EV71	EV71	
	5853/SIN/000009	Intestine	Dead	–	–	–	
	5854/SIN/000009	Spleen	Dead	–	EV71	–	
	5855/SIN/000009	Oral swab	Dead	EV71	EV71	EV71	AF316322
	5858/SIN/000009	Lung	Dead	–	EV71	–	
	5859/SIN/000009	Brain	Dead	–	EV71	EV71	
	5860/SIN/000009	Tonsils	Dead	–	EV71	–	

[a] –, negative result.
[b] ND, not done.
[c] NPA, nasopharyngeal aspirate.

template for subsequent PCR experiments. A 5-μl aliquot of each cDNA template was used in a total reaction volume of 50 μl containing 1× PCR buffer, 0.2 mM deoxynucleoside triphosphates mix, a 0.2 μM concentration each of forward and reverse primers, and 2.5 U of AmpliTaq polymerase (Applied Biosystems, Foster City, Calif.). PCR experiments using various primers were essentially carried out as described earlier (3, 10, 12, 16) in a reaction mixture of 50 μl. The PCR products (10 μl each) were subjected to electrophoresis in agarose gels, with a 100-bp DNA ladder serving as a molecular marker. PCR was performed in batches of 10 to avoid reagent and tube-to-tube cross-contamination. An additional negative control with RNA-free distilled water and a positive control cDNA template of the EV71 reference strain 7423/MS/87 were included.

Seminested PCR amplification. The first-round PCR was carried out using the EV71 type-specific primer pair VP1F2 (5′-GTTCTTAACTCACATAGCA-3′, corresponding to nucleotides 2646 to 2664 of 7423/MS/87) and EV71R2 (5′-TTGACAAAAACTGAGGGGTT-3′, nucleotides 2986 to 2967) under PCR conditions described previously by Singh et al. (12). The second-round seminested PCR was performed using primers VP1F2 and 162A (5′-CCRGTAGGKGTRCACGCRAC-3′, nucleotides 2872 to 2853) (3). The second amplification was carried out under the same conditions as the first, except that 1 μl of the first-round PCR product was added to 49 μl of PCR mixture and a total of 35 cycles were used.

Nucleotide sequence analysis. Purified products were cycle sequenced using the ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit. DNA sequencing was performed using the ABI PRISM 377 DNA sequencer (Applied Biosystems). Homology searches were carried out using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). The percentage of sequence identity was calculated using the Blast 2 Sequences program, which permits alignment of two given sequences by using the BLAST engine for local alignment (13).

Nucleotide sequence accession numbers. The 5′ UTR sequence data for 10 strains derived from nine different patients were deposited in the GenBank database under the accession numbers shown in Table 1.

RESULTS

RT-PCR and nucleotide analyses using pan-enterovirus primers. Since HFMD may be caused by several enterovirus serotypes and due to the urgency of the outbreak involving four fatalities, a pair of universal pan-enterovirus primers (10, 15, 16) was employed in RT-PCR and amplicon sequencing to analyze the enterovirus(es) responsible for the outbreak. Twenty-five specimens from 18 nonfatal cases and 26 specimens from 4 fatal cases were tested. RT-PCR with universal primers successfully generated target bands of 154 bp in 9 of the 25 specimens from the nonfatal cases and in 19 of the 26 specimens from fatal cases. Amplicons showing strong intensity in agarose gels were observed for clinical specimens originating from stool, rectal swabs, and throat swabs. Faint bands were detected for specimens derived from the brain, heart, and lung tissues of the deceased patients. Hence, enteroviruses were successfully detected from 55% (28 out of 51) of the clinical specimens examined by RT-PCR with the universal primers (Table 1). Specimens derived from 41% (9 out of 22) of the patients examined were confirmed to harbor enteroviral RNA sequences by RT-PCR followed by cycle sequencing.

EV71 was detected directly from clinical and autopsy specimens such as the brains, hearts, and lungs of two dead siblings (i.e., patients 21 and 22) who had encephalitis, myocarditis, interstitial pneumonitis, and pulmonary edema. A positive EV71 amplicon was also detected from the rectal swab of another deceased patient, no. 20. RT-PCR failed to generate any positive amplicons from four autopsy samples from deceased patient 19, thus suggesting either the involvement of a virus other than enterovirus or an enteroviral titer too low to allow any detection.

The 154-bp amplicons derived from the 5′ UTRs of 10 specimens were sequenced and compared with sequences of known enteroviral strains isolated elsewhere via computational analysis. As shown in Table 1, one of the strains was genotypically recognized as coxsackievirus A16 or CA16 (patient 2), with 97% nucleotide similarity. Coxsackievirus B4 was genotypically identified in another patient (no. 5), with 94% nucleotide identity. EV71 sequences were identified in eight other clinical specimens from different patients, with the 154-bp amplicons showing 96 to 97% nucleotide similarity to EV71 strains previously isolated in Singapore (12). The enteroviruses detected in specimens derived from three of the fatal cases were also genotypically identified as EV71.

EV71 nucleotide sequences identified in clinical specimens from the two deceased siblings correlated with serotyping data by neutralization assays (Table 1). Twenty-three specimens from which no positive amplicons were generated with the universal primers also failed to produce any cytopathic effect in cell cultures. However, a significant number of specimens (another 21) failed to yield any virus isolate in cell cultures but were positive by RT-PCR with universal primers, thereby substantiating the potential of molecular amplification for detecting a low viral load or even nonviable virions present in clinical specimens.

RT-PCR assay using EV71 type-specific primers. While sequencing of the 154-bp RT-PCR products following amplification of the 5′ UTR with universal pan-enterovirus primers can provide accurate identification of enterovirus types, it is still too time-consuming and costly to sequence large numbers of isolates in outbreak situations. As a significant number of samples from the present outbreak were sequenced and identified to be EV71, we saw an opportunity to evaluate type-specific primers for direct detection of EV71 in clinical specimens. EV71-specific primers that target the VP1 region have shown high specificity and sensitivity for detecting EV71 isolates derived from cell cultures (3, 8, 12, 14). However, there is a scarcity of data available regarding the specificity and sensitivity of these primers for RT-PCR performed directly on clinical material.

To test the efficacy of EV71 type-specific primers, experiments were performed using two primer pairs, 92S-93A and 159S-162A with mixed base composition, designed by Brown et al. (3), as well as VP1F2 and EV71R2, designed by Singh et al. (12). Twenty-seven cell culture isolates were studied, including the prototype EV71/7423/MS/87 strain, 2 EV71 isolates from the recent outbreak, 19 endemic EV71 strains isolated previously in Singapore (12), 3 coxsackievirus A16 strains, and 2 echovirus 30 strains (Table 2). The primer pair VP1F2-EV71R2 generated positive amplicons for 82% (18 of 22) of EV71 isolates. The primer pair 159S-162A yielded positive amplicons for all of the EV71 isolates. Neither primer pair amplified any coxsackievirus A16 or echovirus 30, thus reiterating their high specificity for EV71. The primer pair 92S-93A generated amplicons in only 14% (3 of 22) of the EV71 isolates. However, 92S-93A also amplified CA16 and echovirus 30 and therefore was not completely specific for EV71. The primer pairs VP1F2-EV71R2 and 159S-162A were then further evaluated using 26 representative clinical specimens from HFMD patients from the outbreak (Table 1). Only one (3.8%) specimen from patient no. 13 tested positive by RT-PCR with either the VP1F2-EV71R2 or the 159S-162A primer pair.

TABLE 2. Cell culture isolates tested by single-round RT-PCR with EV71 type-specific primers

Cell culture isolate(s)	No. of samples tested	No. of positive samples with primers:		
		VP1F2-EV71R2[a]	159S-162A[b]	92S-93A[b]
EV71/7423/MS/87 (reference strain)	1	1	1	1
EV71 outbreak strains	2	2	2	2
EV71 endemic strains	19	15	19	0
Coxsackievirus A16	3	0	0	2
Echovirus 30	2	0	0	1

[a] EV71 type-specific primers designed by Singh et al. (12).
[b] EV71 type-specific primers designed by Brown et al. (3).

Development of a seminested PCR assay. RT-PCR with pan-enterovirus primers could detect EV71 in 49% (25 of 51) of the clinical specimens and could detect non-EV71 enteroviruses in 6% (3 of 51) of the specimens, while no viral amplicons could be detected in the remaining 45% (23 of 51) of specimens. The use of pan-enterovirus primers for detecting EV71 was limited by the need to sequence each amplicon. Such an approach is costly and time-consuming. Hence, a seminested PCR was developed using VP1F2-EV71R2 (12) as the outer set of primers and VP1F2-162A as the inner set of primers. Seminested PCR products of 227 bp were amplified in 17 out of 48 tested specimens from the outbreak, i.e., an EV71 detection rate of 35%. The detection of EV71 RNA sequences in clinical specimens by using seminested RT-PCR was further substantiated by EV71 detection in seven additional specimens that were initially negative by cell culture isolation or by RT-PCR with pan-enterovirus primers.

Detection of EV71 by cell culture isolation and neutralization tests exhibited 100% correlation with the RT-PCR data with pan-enterovirus primers. Out of the 17 specimens identified as EV71 by seminested PCR, 4 correlated well with cell culture results, while 10 correlated well with RT-PCR with universal primers. The EV71 detection rates were 35% (17 of 48) for seminested PCR, compared to 49% (25 of 51) for RT-PCR with pan-enterovirus primers and 14% (7 of 51) by cell culture isolation.

Out of the 51 clinical specimens tested for the presence of enteroviruses by the two amplification techniques or by cell culture isolation, no viable nor nonviable enterovirus was detected in 31% (16 of 51) of specimens. Enteroviruses were recovered from only seven clinical specimens, and all were serotyped as EV71 by neutralization tests. No cytopathic changes were observed in the other specimens even after repeated passages in RD and HeLa cells. The remaining 35 specimens were shown to contain viable or nonviable enteroviruses by cell culture and/or RT-PCR methods and were considered true positives for enteroviruses. However, sequencing of the amplicons of RT-PCR with universal primers revealed EV71 sequences in 32 specimens but not in another three. Taking these findings into consideration, the detection rate by RT-PCR using universal primers and sequencing increased to 71% (25 of 35), versus only 20% (7 of 35) by cell culture and neutralization tests (Table 3). Direct detection of EV71 by seminested RT-PCR was successful in 53% (17 of 32) of clinical specimens, while RT-PCR using

TABLE 3. Comparison of diagnostic positivity by cell culture versus RT-PCR assays for the direct detection of EV71 from clinical specimens[a]

Technique	Fraction of true-positive specimens (%)
Cell culture isolation	7/35 (20)
RT-PCR with pan-enterovirus primers (5′ UTR)[b]	25/35 (71)
Seminested PCR with EV71 type-specific primers (VP1)	17/32 (53)
RT-PCR with EV71 type-specific primers (VP1)[c]	1/21 (4.8)
RT-PCR EV71 type-specific primers (VP1)[d]	1/21 (4.8)

[a] A total of 51 clinical specimens were studied. Seminested PCR with EV71-specific primers was performed for only 48 samples, since three non-EV71 samples were excluded. Single-round RT-PCR with EV71 primers was carried out on only 21 selected specimens. True-positive specimens refer to those from which viable or nonviable EV71 was detected by at least one of the methods.
[b] Universal pan-enterovirus primers targeting the 5′ UTR (10, 15, 16).
[c] EV71 type-specific primers targeting the VP1 region described by Singh et al. (12).
[d] EV71 type-specific primers targeting the VP1 region described by Brown et al. (3).

specific primer pairs (3, 12) yielded a very low positivity rate of only 4.8% (1 of 21).

DISCUSSION

Diagnostic testing of clinical specimens from 22 different patients involved in the Singapore outbreak revealed EV71 as the major etiological agent. Enteroviral RNA sequences identified in specimens derived from three of the four fatal cases were genotypically identified as EV71. Viruses isolated from two deceased siblings were also serotypically confirmed as EV71. Coxsackievirus types A16 and B4 were the only two non-EV71 enteroviruses identified in two patients. Hence, EV71 was the predominant serotype involved in the HFMD outbreak in Singapore in 2000. This is of considerable significance in Asia, which has already been plagued by recent major epidemics due to EV71 (6, 7, 11).

The need for accurate and rapid diagnostic tests for EV71 infection is widely appreciated and was fulfilled by the use of the universal enterovirus primer pair for RT-PCR and for sequencing of the 154-bp amplicons. This combination could genotypically identify enteroviruses directly from a broad range of clinical specimens, including autopsy specimens of the brains, hearts, and lungs from two deceased siblings. Out of the 35 true-positive specimens, 71% (25 of 35) were confirmed to be EV71 by cell culture as well as by RT-PCR and sequencing with universal primers. However, one has to consider that many specimens tested for the presence of enteroviruses were from tissues that do not usually support virus replication, and the virus may not be readily detected by RT-PCR. If more samples such as stool, rectal swabs, and throat swabs were collected, the percentage of positive results may be significantly higher.

Both pairs of EV71 type-specific primers, VP1F2-EV71R2 and 159S-162A, were able to detect isolates of the prototype strain 7423/MS/87, endemic EV71 strains isolated previously, and two EV71 strains from the outbreak. Primer pair 159S-162A was more sensitive than VP1F2-EV71R2 and 92S-93A for detecting EV71 strains endemic in Singapore. The mixed

base composition of the primer pair 159S-162A could account for its 100% sensitivity for detecting EV71 isolates compared to the 82% sensitivity of primer pair VP1F2-EV71R2. Although both primer pairs showed relatively high sensitivity for testing isolates of EV71, the sensitivity of RT-PCR with these primers for the direct detection of EV71 in clinical specimens was a very low 5%. Degradation of the viral genome due to the presence of RNases in clinical specimens or an extremely low virus load could explain nondetection. Another possible explanation for this discrepancy is primer-template mismatches arising from genetic diversity of the capsid genes of EV71 strains encountered in the outbreak, despite the primer pair 159S-162A being designed to accommodate base changes.

Conventional PCR is not as sensitive as seminested or nested PCR, and the results may be misleading. Furthermore, seminested PCR offers potentially greater sensitivity in direct testing of clinical specimens (4). The greater sensitivity of seminested PCR could detect EV71 in seven clinical specimens that tested negative either by cell culture or by RT-PCR using universal primers. The primers employed in the newly developed seminested PCR were highly specific, by generating 227-bp amplicons diagnostic for EV71. The target amplicons derived from the VP1 region were authenticated by direct sequencing and displayed 97 to 100% identity to endemic EV71 strains. In the outbreak, our seminested PCR assay proved to be feasible for EV71 detection directly from clinical specimens, with rapid confirmation achievable within a day.

As HFMD outbreaks are often caused by cocirculation of EV71 and CA16 (14), our EV71 type-specific seminested PCR primers can complement the CA16-specific primers designed by Bendig et al. (2) in RT-PCR assays for the rapid detection of these two serotypes in clinical specimens. The prompt recognition of EV71 infections, which in recent years have been associated with more serious illness and higher fatalities than those caused by CA16, will enable immediate intervention and severance of the transmission chain in an epidemic.

Prolonged storage of clinical specimens and delays in their transportation to the laboratory may have resulted in a gradual decline in viral titer and affected the intensity of some amplified products. The enteroviral RNA genome is extremely susceptible to degradation by ubiquitous RNases present in body fluids and tissues. The higher detection by seminested PCR may be attributed to the further amplification of the first PCR product, which was initially below the level of detection. When viral isolation remains negative due to nonviability of viruses in clinical specimens, nested PCR assays are ideal for the molecular identification of enteroviral agents responsible for an outbreak (4). Provided that stringent precautions are adopted to avoid contamination, seminested and nested PCR assays are particularly useful for clinical samples such as cerebrospinal fluid, in which low viral copy numbers may be present (4), thus yielding genetic evidence for the diagnosis of HFMD with neurological involvement.

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