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Detection of H-1 Parvovirus and Kilham Rat Virus by PCR

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H-1 virus and Kilham rat virus (KRV) are autonomous parvoviruses which generally cause subclinical infections in rats and can cause persistent infections in cell cultures. In this study, primer sets specific for either H-1 or KRV were designed on the basis of DNA sequence comparisons of the rodent parvoviruses. The specificities of the H-1- and KRV-specific primer sets were determined by testing viral preparations of seven different parvoviruses and nine other viruses known to infect rodents. The H-1-specific PCR assay amplified the expected 254-bp product only in the presence of H-1 viral DNA and was able to detect as little as 100 fg of H-1 viral DNA. The KRV-specific PCR assay generated the expected 281-bp product only when KRV viral DNA was used as the template and was able to detect as little as 10 pg of KRV viral DNA. Each assay was able to detect its respective virus in tissues from rats experimentally infected with H-1 or KRV. In contrast, no product was amplified by either assay with tissues from mock-infected rats. Our findings indicate that these PCR assays provide rapid, specific, and sensitive methods for the detection of H-1 or KRV infection in rats and cell culture systems.

H-1 parvovirus and Kilham rat virus (KRV) were first identified in the late 1950s as contaminants of tumor cell lines which had been passaged in rats (24, 34). Natural infection of rats with either of these viruses is common and is generally subclinical. Clinical illness has been observed in only a few cases of natural infection with KRV and has not been observed in cases of natural infection with H-1. Clinical signs associated with natural KRV infection include fetal resorption in dams, runting, ataxia, cerebellar hypoplasia, and jaundice in suckling rats and sudden death, scrotal cyanosis, abdominal swelling, and dehydration in juvenile rats (9, 21). KRV can also produce persistent infection in rats. Neonatal rats infected with KRV and individually housed at weaning were shown to carry the virus persistently for up to 6 months (19). Persistence of H-1 viral infection in rats has not been adequately studied; however, both H-1 and KRV have been shown to cause persistent infection in cell cultures (2, 14). The ability of H-1, KRV, and other parvoviruses to produce persistent infections coupled with the remarkable resistance of the viruses to environmental conditions has resulted in a high frequency of contamination of cell cultures and transplantable-tumor systems (10, 13, 16).

In addition to contaminating cell cultures and transplantable-tumor systems, H-1 and KRV may interfere with research in other ways. H-1 virus has been shown to inhibit experimental tumor induction in hamsters (35, 36) and can cause hepatocellular necrosis in infected rats that have been subjected to hepatotoxic chemicals or parasitism (23, 25). KRV has been reported to alter lymphocyte responses and cytotoxic lymphocyte activity in vitro (7, 8, 12) and can induce interferon production in vivo (20). KRV may also compromise studies of fetal development and teratogenesis since it can cross the placenta and cause cerebellar hypoplasia, hepatitis, and death in rat fetuses (18). Because of these detrimental effects on research, rats and cell culture systems are routinely screened for H-1 and KRV.

There are several methods for diagnosing H-1 or KRV infection in rats and biological materials. Serologic assays are at present the most common approach to diagnosis of H-1- and KRV-infected rats. An enzyme-linked immunosorbent assay (ELISA) or indirect fluorescent-antibody assay is generally used as the screening assay, while a hemagglutination inhibition assay is commonly used as the confirmatory test (25). The ELISA and indirect fluorescent-antibody assay are sensitive but lack specificity because of antibodies that cross-react with nonstructural proteins that are conserved among the rodent parvoviruses. The hemagglutination inhibition assay is specific but lacks sensitivity, since it detects only antibody directed against the viral hemagglutinin. These serologic assays are able to identify rat colonies in which infection with H-1 or KRV is endemic but are unable to detect outbreaks of acute infection in which seroconversion has not occurred (33). Histopathology may be used to diagnose the rare clinical outbreaks of KRV infection in rat colonies, but it is not useful for detection of subclinical infections. Virus isolation is considered the "gold standard" for detection of viral infection and has been useful for detection of persistent viral infections with KRV (27), but the method is expensive and labor-intensive and has a slow turnaround time. Recently, a PCR assay was developed to identify rats acutely infected with KRV (33). This assay was able to identify rats infected with KRV but was unable to distinguish between viral preparations of KRV and H-1. Detection of H-1 or KRV contamination of biological materials currently relies upon virus isolation or the rat antibody production (RAP) test. The basis for RAP testing is the induction of antibody against a contaminating virus when the biological material is inoculated into a rat. Serum from the rat is then screened by serologic assays currently used to diagnose rat viral infections. RAP testing has a slow turnaround time and is inherently prone to the disadvantages of the serologic assays currently used to diagnose rodent parvovirus infections. Given the limitations of the available diagnostic methods, a rapid, direct method for the specific detection of H-1 and KRV parvoviruses in infected animals and biological materials is needed.

In this study, gene amplification was investigated as a method for specific diagnosis of H-1 and KRV infections in rats. Primer sequences were selected from unique regions of

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TABLE 1. Parvovirus isolates used for determination of the specificities of the H-1- and KRV-specific PCR assays

Isolate	Host	Reference
H-1	Rat	34
KRV	Rat	24
MOPV1	Mouse	26
HOPV	Hamster	15
LuIII	Unknown	16
MVM(i)	Mouse	6
MVM(p)	Mouse	11

the H-1 and KRV capsid genes to provide primer sets specific for H-1 and KRV, respectively. These primers were then synthesized, and a DNA amplification procedure was developed to identify viral DNA in infected cell cultures and in tissues from rats experimentally infected with H-1 or KRV. The results obtained indicate that these PCR assays provide rapid, specific, and sensitive methods for identification of H-1- and KRV-infected rats and cell culture systems.

MATERIALS AND METHODS

Viral isolates and propagation. The rodent parvovirus isolates used in this study are listed in Table 1. H-1 and LuIII parvoviruses were grown in 324K simian virus 40-transformed newborn human kidney cells (30), KRV was grown in rat glial tumor cells (C_6 Glial, ATCC CCL 107), mouse orphan parvovirus 1 (MOPV1) was grown in murine cytotoxic T cells (CTLL-2, ATCC TIB 214), hamster orphan parvovirus (HOPV) was grown in baby hamster kidney cells (BHK-21, ATCC CCL 10), an immunosuppressive strain [MVM(i)] of minute virus of mice was grown in murine T lymphoma cells (S49.1TB.2, ATCC TIB 30), and a prototype strain [MVM(p)] of minute virus of mice was grown in murine $A9_{2L}$ fibroblasts (32). MOPV1, also referred to as Fitch orphan parvovirus and mouse parvovirus 1, was originally isolated as a contaminant of murine T cells (26). HOPV is a novel parvovirus isolated from an infected hamster by our laboratories (5, 15). All cells were grown in Dulbecco's modified Eagle's medium (Hazleton, Lenexa, Kans.) containing 10% Serum-plus (JRH Biosciences, Lenexa, Kans.) at 37°C in a 10% CO_2 atmosphere, except for CTLL-2 cells, which were grown in RPMI medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% Serum-plus, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid), 2 mM sodium pyruvate, and 10 U of human recombinant interleukin-2 (Gibco BRL, Grand Island, N.Y.) per ml of medium.

The median (50%) tissue culture infective dose ($TCID_{50}$) for each viral stock was determined in 96-well microtiter plates with seeding densities of 2.5×10^2 cells per well for CTLL-2 cells and 5×10^3 cells per well for all other cell lines. Concentrated viral preparations were then obtained by infecting mammalian cells at a multiplicity of infection of 0.1 and incubating cultures at 37°C. Cell pellets were collected by centrifugation (10 min at $500 \times g$) when approximately 90% of the cells exhibited cytopathic effect. Each cell pellet was resuspended in a 1/10 volume of Tris-EDTA (50 mM Tris, 10 mM EDTA, pH 8.5) and subjected to four freeze-thaw cycles. Cellular debris was then removed by centrifugation (10 min at $1{,}000 \times g$). The DNA content of each viral preparation was determined by dot blot quantitation (29), and the preparations were diluted to 20 μg of DNA per ml to equilibrate the DNA template concentration for the PCR assays.

Oligonucleotide primers. Oligonucleotide primers (Table 2) were synthesized at the DNA Core Facility, University of Missouri, Columbia. Sequences were selected on the basis of sequence alignments generated by using the EuGene software package (Baylor College of Medicine, Houston, Tex.). All sequence data were obtained from GenBank with the exception of the sequences for MOPV1 and HOPV, which were determined in our laboratories (4), and the sequence for KRV (kindly provided by Lisa Ball-Goodrich, Yale University, New Haven, Conn.). Primers were designed from segments of H-1 or KRV exhibiting maximum heterology with all other rodent parvoviruses.

PCR amplification. All reactions were performed in a 50-μl volume in an automated Perkin-Elmer model 9600 thermocycler. Each reaction mixture contained various amounts of template DNA (see below), 1 μM each oligonucleotide primer, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), and 2.0 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.). PCR consisted of 30 s of denaturation at 94°C followed by 35 cycles of 2 s of denaturation of 94°C, 2 s of annealing at 55°C, and 30 s of elongation at 72°C. PCR products (10 μl) were electrophoretically separated in a 3% NuSieve agarose gels (FMC BioProducts, Rockland, Maine), stained with ethidium bromide, and visualized by UV light. DNA markers of known sizes were run on each gel to facilitate determination of the sizes of the reaction products.

To test the specificity of each assay, PCR assays were performed with 100 ng of template DNA from preparations of H-1, KRV, MOPV1, HOPV, LuIII, MVM(i), and MVM(p). The specificity of each assay was also evaluated with viral stocks of K virus, lymphocytic choriomeningitis virus, mouse adenovirus 2, mouse cytomegalovirus, mouse hepatitis virus, polyomavirus, pneumonia virus of mice, reovirus 3, and Sendai virus, all of which were obtained from the University of Missouri Research Animal Diagnostic and Investigative Laboratory (Columbia).

To test the sensitivity of the PCR assay, 10-fold serial dilutions of H-1 or KRV DNA, ranging from 100 ng to 1 fg, were used as templates in their respective PCR assays. To simulate diagnostic conditions, assays were performed in the presence of 1.25 μg of DNA extracted from the kidney of a Sprague-Dawley rat from a colony serologically negative for H-1 and KRV.

Animal infections. Three midgestation rats were obtained from a colony determined to be free of H-1 and KRV infection by repeated serologic testing (Harlan Sprague-Dawley, Indianapolis, Ind.). The rats were housed in microisolator cages in accordance with the *Guide for the Care and Use of Laboratory Animals* (26a). Six 2-day-old rats from each litter were inoculated with 10-μl volumes containing either 10^4 $TCID_{50}$s of H-1 (given intraperitoneally), 10^4 $TCID_{50}$s of KRV (given oronasally), or Tris-EDTA (given oronasally). Viral inocula and routes of administration were chosen on the basis of previously reported studies (17, 22). Seven days postinoculation, rats were euthanized and tissues from the liver, kidneys, spleen, testicles or uterus, intestine, and brain were collected from each rat and frozen at −80°C.

DNA isolation. DNA was extracted from rat tissues with a QiAmp tissue kit (Qiagen Inc., Chatsworth, Calif.) by following the manufacturer's instructions. The DNA content and the purity of the tissue DNA extracts were determined by measuring the A_{260}/A_{280} optical density ratio with a Perkin-Elmer Lambda 3B UV-visible spectrum spectrophotometer. The amount of DNA used as the template in PCR testing of tissues from experimentally infected and mock-infected rats was 1.25 μg.

DNA sequencing. PCR products amplified from rat tissues were purified on 3.5% polyacrylamide gels, and the sequences were determined by the *Taq* dideoxy-chain termination method with a commercially available kit (*Taq* dye deoxy terminatory cycle sequencing kit; Applied Biosystems, Inc., Foster City, Calif.). Sequence data were analyzed with the EuGene software package.

RESULTS

Evaluation of PCR primers specific for H-1 and KRV. Comparisons of the nucleotide sequences of H-1 and KRV with those of other autonomous parvoviruses revealed several areas of limited homology. On the basis of these comparisons, primer sets specific for H-1 (3479f and 3732r) and KRV (3691f and 3971r) (Table 2) were designed to provide maximum heterology with the aligned regions from the other rodent parvo-

TABLE 2. Oligonucleotide primers used for H-1- and KRV-specific PCR assays

Primers	Sequence (5′–3′)	Position (5′–3′)[a]
H-1 specific		
3479f	CTAGCAACTCTGCTGAAGGAACTC	3479–3502
3732r	TAGTGATGCTGTTGCTGTATCTGATG	3732–3707
KRV specific		
3691f	GCACAGACAACCAAACAGGAACTCTCC	3691–3717
3971r	AGTCTCACTTTGAGCGGCTG	3971–3952

[a] The position within the H-1 or KRV viral genomic sequence that corresponds to the 5′ and 3′ ends of each primer.

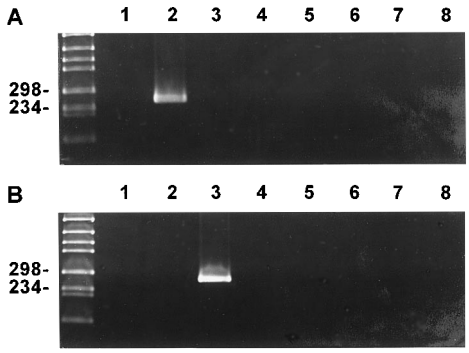

FIG. 1. Ethidium bromide-stained agarose gel demonstrating the specificities of the H-1-specific PCR assay (A) and the KRV-specific PCR assay (B) when used with other rodent parvoviruses. Shown are PCR products resulting from amplification with 100 ng of viral DNA as the template. Lanes: 1, no-template control; 2, H-1; 3, KRV; 4, MOPV1; 5, HOPV; 6, LuIII; 7, MVM(i); 8, MVM(p). The migration of molecular size markers is shown in the left lane of each gel. Sizes are in base pairs.

viruses and were named according to the genomic sequence position of the nucleotide at the 5′ end of each primer.

The specificities of the H-1- and KRV-specific primer sets were determined. The primer sets were tested against preparations of parvoviruses, including H-1, KRV, MOPV1, HOPV, LuIII, MVM(i), and MVM(p), and preparations of other viruses that infect rodents, including K virus, lymphocytic choriomeningitis virus, mouse adenovirus 2, mouse cytomegalovirus, mouse hepatitis virus, polyomavirus, pneumonia virus of mice, reovirus 3, and Sendai virus. The H-1-specific primer set amplified only H-1 viral DNA and produced the expected 254-bp product (Fig. 1A). Likewise, the KRV-specific primer set amplified only KRV DNA and produced the expected 281-bp product (Fig. 1B).

The sensitivities of the PCR assays were determined by amplifying 10-fold serial dilutions of H-1 or KRV DNA in the presence of 1.25 μg of DNA isolated from the kidney of an uninfected rat. The H-1-specific PCR assay detected a 254-bp product when as little as 100 fg of H-1 viral DNA was used as a template (Fig. 2A). The KRV-specific PCR assay detected the expected 281-bp product when as little as 10 pg of KRV viral DNA was used as a template (Fig. 2B).

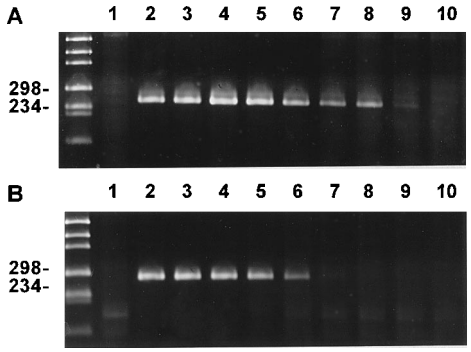

FIG. 2. Ethidium bromide-stained agarose gel demonstrating the sensitivities of the H-1- and KRV-specific PCR assays. Shown are PCR products resulting from amplification of serial dilutions of H-1 (A) and KRV (B) viral DNA in the presence of kidney DNA from an uninfected rat. Lanes: 1, kidney DNA only; 2, 100 ng of viral DNA; 3, 10 ng of viral DNA; 4, 1 ng of viral DNA; 5, 100 pg of viral DNA; 6, 10 pg of viral DNA; 7, 1 pg of viral DNA; 8, 100 fg of viral DNA; 9, 10 fg of viral DNA; 10, 1 fg of viral DNA. The migration of molecular size markers is shown in the left lane of each gel. Sizes are in base pairs.

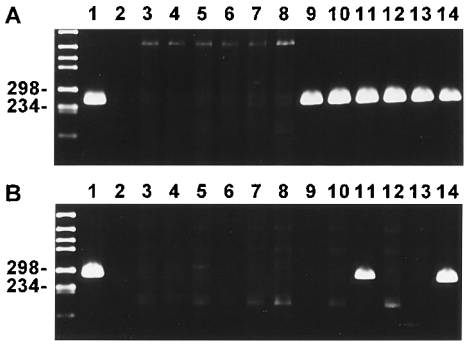

FIG. 3. PCR amplification of kidney DNA from mock-infected rats or rats experimentally infected with H-1 or KRV. (A) PCR products resulting from the amplification of kidney DNA from mock-infected rats (lanes 3 to 8) and H-1-inoculated rats (lanes 9 to 14) by using the H-1-specific PCR assay. (B) PCR products resulting from the amplification of kidney DNA from mock-infected rats (lanes 3 to 8) and KRV-inoculated rats (lanes 9 to 14) by using the KRV-specific PCR assay. Lane 1 is the positive control and lane 2 is the no-template control for each assay. The migration of molecular size markers is shown in the left lane of each gel. Sizes are in base pairs.

PCR of DNA from tissue samples. DNA amplifications with the H-1- or KRV-specific primer set were performed on DNA extracted from tissues of experimentally infected and mock-infected rats. The H-1-specific PCR assay amplified the expected 254-bp product in tissues from six of six rats experimentally infected with H-1 virus when kidney or intestinal DNA was used as a template (Fig. 3A). DNA extracts of liver, spleen, uterine, and brain tissues from two of the H-1 infected rats were also tested by the H-1-specific PCR assay, and the 254-bp product was amplified from each of these tissues from both rats (data not shown). The KRV-specific PCR assay detected the expected 281-bp product in tissues from two of six rats infected with KRV virus when kidney or intestinal DNA was used as a template (Fig. 3B). DNA extracts of the liver, spleen, uterine, and brain tissues from the two KRV-positive rats were also tested by the KRV-specific PCR assay, and the 281-bp product was amplified from each of these tissues from both rats (data not shown). No PCR products were detected by either assay in tissues from any of the six mock-infected rats when kidney or intestinal DNA was used as a template (Fig. 3A and B) or when DNA extracts from liver, spleen, uterine, or brain tissues from two of the mock-infected rats were used as a template (data not shown).

The H-1- and KRV-specific PCR products detected in tissues from the experimentally infected rats were sequenced to confirm the identities of the products as H-1 and KRV sequences, respectively. The sequence for each product aligned exactly with the capsid region corresponding to the primer-encompassed region for each virus (data not shown).

DISCUSSION

In this study PCR assays were developed to detect H-1 and KRV viral infections. Two oligonucleotide primer sets, one specific for H-1 and one specific for KRV, were designed and synthesized on the basis of nucleotide sequence comparisons of prototypic and recently identified rodent parvoviruses. Each assay was evaluated for specificity to its respective virus by testing the primer sets against a panel of parvoviruses and a panel of other viruses that infect rodents. The 254-bp product expected for the H-1-specific assay was produced only when H-1 viral DNA was present and the 281-bp product expected for the KRV-specific assay was produced only when KRV

DNA was present, indicating that each assay was specific for its respective virus. The specificity of each assay for its target viral DNA was also confirmed by testing DNA extracts from rat tissues. Each assay was able to detect its respective viral DNA in tissues from experimentally infected rats, yet neither assay amplified the expected PCR product from tissues of mock-infected rats.

In this study the expected PCR product was detected when as little as 100 fg of DNA from the H-1 viral preparation was used as the template for the H-1-specific assay or when 10 pg of DNA from the KRV viral preparation was used as the template for the KRV-specific assay. The 100-fold difference in sensitivity between the two assays appears to be due to the concentration of viral DNA in the template, since a 100-fold difference in sensitivity was also observed when the same two templates were used in a PCR assay that detects all autonomous rodent parvoviruses (data not shown). The latter PCR assay utilizes primers designed on the basis of the segments of the viral genome that are identical among MVM, H-1, and KRV; therefore, the same level of sensitivity would be expected with each virus when equivalent amounts of viral DNA are used as the template (3).

Both the H-1- and KRV-specific PCR assays were able to detect viral DNA in tissues from experimentally infected rats. The H-1-specific assay was able to detect target viral DNA in tissues from six of six rats infected with H-1 virus. However, the KRV-specific assay was able to detect target viral DNA in tissues from only two of six rats infected with KRV. There are several reasons which may account for the detection of KRV in tissues from only two of six rats. The oronasal route of inoculation and dose of KRV (10^4 $TCID_{50}$s per rat) were chosen on the basis of a previous study of the KRV-Yale strain, originally isolated during a naturally occurring outbreak of KRV in a colony of Crl:COBS,CD juvenile rats (9). In the study KRV-Yale was passaged twice in cell culture before rats were experimentally infected with the virus (17). The Kilham strain of KRV used in our study was originally isolated from tissues of tumor-bearing rats (24) and has been passed in cell culture many times. The infectivity and pathogenesis of the Kilham strain of KRV in neonatal Sprague-Dawley rats may be different from those of the KRV-Yale strain because of the differences in origin and cell culture passage. Additionally, since Sprague-Dawley rats are an outbred stock, their inherent susceptibility to KRV may vary from animal to animal. Any or all of these viral and host factors may account for the low number of PCR-positive rats in our study.

H-1, KRV, and MVM have been extensively characterized and are considered the prototypic rodent parvoviruses. Several other rodent parvovirus strains have recently been identified, including several variants of a mouse isolate, MOPV (1, 5, 26, 31), and a hamster isolate, HOPV (5, 15). Serologic evidence also suggests the existence of an uncharacterized parvovirus in rats that is distinct from the H-1 and KRV serogroups (28, 37). Antibodies induced against these recently identified parvoviruses cross-react with the proteins of prototypic parvoviruses currently used in conventional serologic assays and confound interpretation of serologic results (3). Alternative assays capable of distinguishing the rodent parvoviruses from each other are needed for definitive diagnosis of parvovirus infections. A PCR assay recently developed to detect KRV infection in rats was unable to distinguish between KRV and H-1 (33). The PCR primers utilized in the study were based upon the DNA sequence at the 3′ terminus of the parvovirus genome, a region that is conserved among rodent parvoviruses, and therefore it is not surprising that both H-1 and KRV sequences were amplified by these primers. In the present study, the H-1- and KRV-specific primer sets were based upon sequences from the capsid gene of each virus that are highly divergent with the aligned sequences of other rodent parvoviruses. PCR assays with these H-1- and KRV-specific primer sets allowed definitive identification of H-1 or KRV from other prototypic and recently identified rodent parvoviruses.

The H-1- and KRV-specific PCR assays may also be utilized to detect H-1 or KRV contamination of biological materials. Detection of H-1 or KRV contamination of biological materials currently relies upon the RAP test or virus isolation. RAP testing requires at least 2 weeks to allow for seroconversion and is inherently prone to the disadvantages of the serologic assays currently used to diagnose rodent parvovirus infections. Virus isolation is expensive and labor-intensive and has a slow turnaround time, and therefore, it has limited value as a diagnostic procedure. PCR is rapid, specific, sensitive, and independent from the immune response and therefore is an ideal diagnostic method for detecting viral contamination of biological materials. The specificity and sensitivity of the H-1- and KRV-specific PCR assays reported here indicate that these assays should be useful for detecting contamination of biological materials by H-1 and KRV.

In summary, PCR assays specific for H-1 or KRV were able to distinguish their respective viruses from other prototypic and recently identified rodent parvoviruses, as well as from other rodent viruses. The results of this study indicate that these PCR assays may be useful as diagnostic tools for rapid detection of H-1 or KRV in acutely infected rats and for detection of H-1 or KRV contamination of cell culture systems.

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