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Diagnostic Polymerase Chain Reaction Assays for Identification of Murine Polyomaviruses in Biological Samples

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Purpose: **Mouse polyoma virus and K virus are murine polyomaviruses frequently used in carcinogenicity and cellular biology studies in mice. These viruses can cause persistent infections, which increase the likelihood of transmission through transplantation of cells from infected mice. To identify polyomavirus-infected biological samples, several diagnostic polymerase chain reaction (PCR) assays were developed.**

Methods: **Polyomavirus-family and virus-specific PCR assays were designed and optimized for specificity and sensitivity. The generic (polyomavirus-family) PCR assay and mouse polyoma virus-specific assays were compared with the mouse bioassay for diagnosis of infected cellular samples.**

Results: **Specificity of the PCR assays was confirmed by testing a battery of other murine viruses. The mouse polyoma virus PCR test was the most sensitive assay, detecting as few as 2,000 copies of homologous virus. The K virus PCR assay was about eightfold less sensitive, and the generic PCR test was the least sensitive. Mouse polyoma virus and generic PCR assays amplified mouse polyoma virus in the inoculum and tissues from experimentally infected mice, and performed better than did the mouse bioassay.**

Conclusions: **Results of this study confirm that PCR is a specific and sensitive method for detection of murine polyomaviruses in biological samples.**

Mouse polyoma virus and K virus are taxonomically related but antigenically distinct members of the subfamily *Polyomavirinae* of the family *Papovaviridae.* As a group of viruses, they are frequently referred to as murine polyomaviruses, a term that will be used in this report to represent both viruses. Each virus was isolated as a consequence of its ability to persistently infect mice. Mouse polyoma virus was first identified as a contaminant of filtered mouse leukemic extracts (1), and was proven not only to induce persistent infections but also tumors in mice experimentally infected as neonates (2). The K virus, which was recovered from asymptomatic mice in retrovirus studies (3), caused interstitial pneumonia in infected mouse neonates (4) and persistent infections in adults (5). Mice experimentally and persistently infected with either of these viruses have proven to be valuable research tools in defining the biological effects of virus proteins on cell cycle regulation and transformation (6), and for studying the pathogenesis of disease as models for human papovaviral infections (7, 8). However, viral persistence has also led to inadvertent infection of mice or mouse cells when transplantable reagents (cells or tumors) were contaminated with a murine polyomavirus (9, 10). Undesirable outcomes of such contamination are nullification of data derived from use of infected materials, lost time, and wasted research funds.

The asymptomatic course of the persistent infection and the risk of transmission from passage of infected transplantable cells have prompted use of diagnostic tests to detect the presence of virus in infected mice or mouse tissues. The most commonly used diagnostic test is the mouse antibody production (MAP) test, in which cellular material is injected into immune-naïve mice and antibody to contaminating virus(es) is detected by use of an enzyme-linked immunosorbent assay (ELISA) or an immunofluorescent antibody (IFA) test (11). For the MAP test, it is standard procedure to use juvenile to young adult mice for sample inoculation, which would minimize concerns of murine polyomavirus-induced clinical disease or limited antibody production, which occurs when mice are infected with mouse polyoma virus as neonates (12). Less commonly used diagnostic tests are based on virus detection, either by virus isolation or tissue localization of viral proteins or genomes. Mouse polyoma virus strains propagate readily in several established cell lines, but K virus replicates poorly in either established or primary cells (13). The costly and time-consuming virus isolation methods have been replaced in part by molecular methods, including DNA hybridization and amplification. Of these methods, DNA amplification via polymerase chain reaction (PCR) analysis has been readily used for diagnostic purposes because of the ability to perform it rapidly and the good sensitivity and specificity associated with it (14, 15).

We decided to exploit the advantages of PCR technology in a diagnostic test to screen mouse cells for murine polyomaviruses. We developed a series of murine polyomaviral PCR assays and compared them with antibody production for diagnosis of mouse polyoma virus-infected biological samples. These PCR assays offer rapid results (one to two days versus four weeks for antibody tests), increased sensitivity, and replacement of an animal-based test. Cells from virus cultures and from mice experimentally infected with mouse polyoma virus were used to validate the PCR assays. The generic murine polyomavirus-family and mouse polyoma virus-specific PCR assays proved to be more sensitive than the antibody production test in detecting mouse polyoma vi-

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rus-infected cells. These diagnostic PCR assays offer rapid and sensitive viral screening of biological samples to prevent use of murine polyomavirus-contaminated tissues and animals in research.

Materials and Methods

Viral propagation and quantitation. Mouse polyoma virus (VR-252 American Type Culture Collection or ATCC, Manassas, Va.) was grown in mouse fibroblast 3T6 (CRL-6319, ATCC) cell line in Dulbecco's modified Eagle's medium (Hazelton Laboratories, Lenexa, Kans.) supplemented with 10% fetal bovine serum at 37°C in a 10% carbon dioxide atmosphere. Mouse polyoma virus was quantitated by plaque assay using standard methods (16). Briefly, 3T6 cells in 100-mm culture plates were inoculated with tenfold dilutions of virus suspensions, and allowed to incubate for 1.5 h. Cultures were washed of non-adherent virus, and overlaid with a low-melt agarose (FMC Bioproducts, Rockland, Maine) containing neutral red dye. Cultures were incubated for 5 to 7 days, and plaques were counted. Mouse polyoma virus titer was determined to be 10^7 plaque-forming units (PFU)/ml.

Cloned K virus (45028, ATCC), and mouse polyoma virus (45018, ATCC) were propagated in bacterial cultures, using standard techniques (17), and plasmid DNA was purified, using a Qiagen Plasmid Purification Kit (Qiagen Inc., Chatsworth, Calif.).

Oligonucleotide primers. Oligonucleotide primer sequences (Table 1) were selected from the GenBank on the basis of sequence alignments of mouse polyoma virus and K virus generated by computer software (Genetics Computer Group, Madison, Wis.). Oligonucleotide primers selected from consensus regions of both viruses were designed for a "generic" assay (i.e., an assay capable of recognizing both murine polyomaviruses known to occur in mice). Primers from regions exhibiting maximum divergence between mouse polyoma virus and K virus and among other viruses and structural protein genes were designed for virus-specific assays. Oligonucleotides were synthesized by GIBCO BRL Custom Primers (Gaithersburg, Md.).

Polymerase chain reaction assays. All reactions were performed in a 50-μml volume in an automated thermocycler (Genamp PCR System 2400, PE Biosystems, Norwalk, Conn.). Each reaction mixture contained approximately 0.1 μg of template, 1.5 μM each primer, 10 m*M* Tris-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, and 50m*M* KCl, 200 μ*M* each dNTP (dATP, dCTP, dTTP, dGTP), 25 μ*M* $MgCl_2$, and 2.50 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.). The PCR cycle parameters consisted of a 5-min denaturation at 94°C followed by 40 cycles of denaturation at 94°C for 2 sec., annealing at primer pair-specific temperatures for 3 sec., and extension at 72°C for 45 sec. The annealing temperatures were 41°C for the generic primers, 57°C for the mouse polyoma virus-specific primers, and 53°C for the K virus-specific primers. The PCR products were electrophoretically separated in 2% NuSieve agarose gels (FMC Bioproducts, Rockland, Maine), stained with ethidium bromide, and visualized with UV light. The DNA markers of known sizes were run on each gel to assist in determining reaction product sizes.

The specificity of each of the three PCR assays was evaluated with 100 ng of template DNA from minute virus of mice, ectromelia virus, mouse parvovirus, and mouse cytomegalovirus and cDNA from lymphocytic choriomeningitis virus (Research Animal Diagnostic and Investigative Laboratory or MU RADIL, University of Missouri, Columbia, Mo.).

To test the sensitivity of each PCR assay, reactions were run with tenfold serial dilutions (starting with 15 ng to 21 ng) of DNA extracted from cloned mouse polyoma virus and K virus. To simulate diagnostic conditions, PCR reactions were performed with 1.0 μg of DNA extracted from the kidney of a BALB/c mouse (Frederick Cancer Research Development Center, Frederick, Md.) that was seronegative for mouse polyoma virus and K virus.

Sequencing of DNA. The PCR products amplified from DNA extracted from mouse polyoma virus and from cloned K virus were removed from a low-melt agarose gel (FMC Bioproducts), and purified, using QIAquick PCR Purification Kit (Qiagen, Inc.) per manufacturer's instructions. Each purified PCR product was used as a template in sequencing reactions, using the *Taq* dideoxy-chain termination method (Taq DyeDeoxy-Terminator cycle sequencing kit, PE Biosciences, Inc., Foster City, Calif.). Reaction products were sequenced at the University of Missouri DNA Core Facility. Sequence data were analyzed, using the GCG software program.

Experimental infection studies. Animal infection studies were approved by the institutional Animal Care and Use Committee. Twenty-four 5-week-old female mice (12 BALB/cAnNCr, and 12 NCR:nude) were obtained from colonies free of mouse polyoma virus and K virus infections (Frederick Cancer Research Development Center). Mice were housed by genotype in sterile static microisolator cages in a room with a 12/12-h light/dark cycle. Room temperature was maintained at 20-21°C, and relative humidity ranged between 50 and 60%. BALB/c and nude mice are mouse strains most frequently used in experimental infection or cancer studies, and provided the opportunity to compare diagnostic approaches for transiently infected mice that produce antibody (BALB/c) with persistently infected mice without antibody (nude). Six animals of each genotype received 10^7 PFU mouse polyoma virus by intraperitoneal injection. The remaining animals were sham-inoculated with cell culture medium. Virus-infected mice were housed in an infectious disease barrier, and sham-inoculated mice were housed in a clean barrier. At 14 and 28 days after inoculation, three infected mice and three control mice from each strain were euthanized with an overdose of carbon dioxide. Blood was collected from BALB/c

Table 1. Oligonucleotide primers for murine polyomavirus-family and virus-specific polymerase chain reaction (PCR) assays

Primers	Sequence (5'-3')	Product Size	Position (5'-3') [a,b]
Generic			
PKf	AAG TAI CTT GTI TTC TCA TT	794-bp polyoma	3379 polyoma/3041 K
PKr	GAC TGG ATG CTT CCT TTA	747-bp K virus	4173 polyoma/3788 K
Polyoma virus			
PVf	TGA TGC CAG GTG CCT AGT AC	272 bp	602
PVr	CAT CGT GTA GTG GAC TGT GG		874
K virus			
Kf	CCA CTC CAT CAT AAA TCC	282 bp	2713
Kr	ACT AAC ACT ACT ACC ACA ATC C		2995

[a]Mouse polyoma virus nucleotide sequence from GenBank accession No. j02289.
[b]K virus nucleotide sequence from GenBank accession No. m57473.

mice, and serum was frozen at -20°C until assayed for polyoma virus antibody by use of the ELISA (MU RADIL). Kidney, heart, mammary gland, skin, and liver were collected from all infected mice and stored at -80°C until processed for PCR analysis.

Isolation of DNA from infected cells. The DNA was extracted from infected 3T6 cells and mouse tissues by use of a QiAmp tissue kit (Qiagen, Inc.) following the manufacturer's protocol. The DNA content and purity of the extracts were determined by measuring the A260/A280 optical density ratio, using a spectrophotometer (Perkin-Elmer Corporation, Norwalk, Conn.).

Results

Comparison of aligned sequences of mouse polyoma virus and K virus revealed limited consensus regions. Degenerate primers for the generic murine polyomavirus-family PCR test were selected from the coding region of VP1 capsid genes of each virus (Table 1). The predicted size of the mouse polyoma virus amplicon was 47 bases larger than that of the K virus amplicon. Virus-specific primers were selected from the VP2 protein-coding region of mouse polyoma virus and from the VP1 open reading frame of K virus (upstream from the consensus regions).

The specificities of the PCR assays were confirmed when assays were run with template DNA from mouse polyoma virus, K virus, and several other viruses that have been reported to contaminate transplantable cells, including minute virus of mice, mouse parvovirus, ectromelia virus, mouse cytomegalovirus, and lymphocytic choriomeningitis virus (Figure 1). The generic PCR assay generated the expected 794-bp product from mouse polyoma virus template, and the 747-bp product from the K virus template. The mouse polyoma virus-specific primers amplified only mouse polyoma virus DNA, producing the expected 272-bp product. The K virus-specific primers produced the expected 282-bp product only with K virus template. Amplicons were not generated when DNA or cDNA from other murine viruses was used as template in any of the PCR assays.

The sensitivities of the PCR assays were determined by amplifying tenfold serial dilutions of mouse polyoma virus or K virus DNA in the presence of kidney DNA extracted from uninfected BALB/c mice (data not shown). The generic PCR assay detected the expected 794-bp product with no less than 21 ng of mouse polyoma DNA, whereas the 747-bp product was detected with no less than 15 pg of K viral DNA. The mouse polyoma virus-specific assay detected the 272-bp product with as little as 21 fg of mouse polyoma viral template DNA, which calculates to represent about 2,000 copies of the mouse polyoma virus genome. The K virus-specific assay detected the 282-bp product when 150 fg of K viral DNA was used as template, which represents about 16×10^4 copies of the virus.

Amplicons from the generic and each of the virus-specific PCR assays were sequenced and analyzed to confirm the identities of the products as mouse polyoma virus and K virus sequences. The K virus and mouse polyoma virus sequences from the generic PCR assay were 98 to 100% identical to Genbank sequences. Amplicons from the virus-specific PCR assays were 100% identical with the respective Genbank viral sequences (data not shown).

Sera obtained from infected BALB/c mice on days 14 and 28 were tested by ELISA for antibodies to mouse polyoma virus. All six serum samples had mouse polyoma virus antibody, with ELISA absorbance values that ranged from 0.074 to 0.145; baseline absorbance for a positive test result is > 0.320. Results for these animals, although they were infected with mouse polyoma virus, would have been interpreted as negative by serologic examination. Control mice had ELISA absorbance values < 0.005.

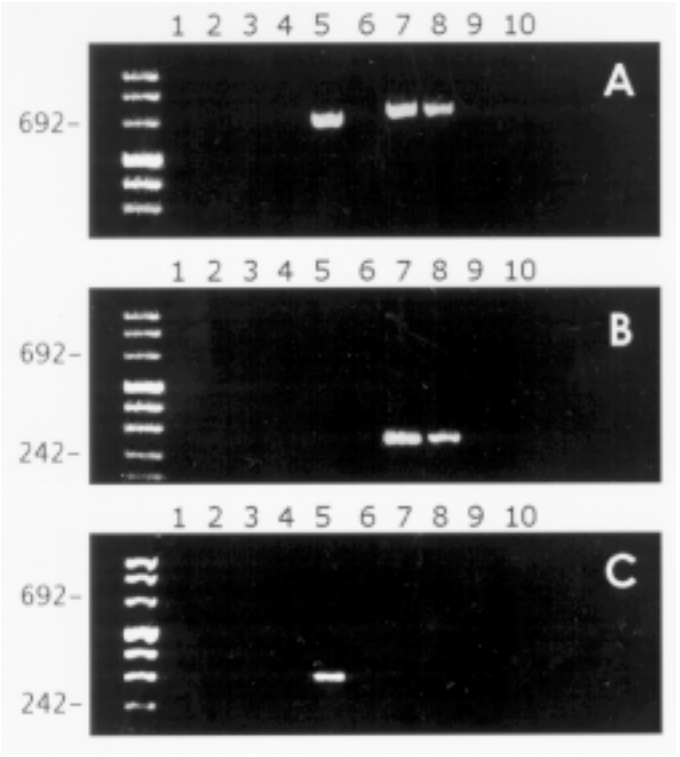

Figure 1. Ethidium bromide stained agarose gel revealing the specificities of the generic (polyomavirus family) PCR (A), the mouse polyoma virus-specific PCR (B) and the K virus-specific PCR (C) when used with template from other murine viruses. Shown are PCR products resulting from amplification with 100 ng of viral DNA as template. Lanes: 1, ectromelia virus; 2, mouse cytomegalovirus; 3, min virus of mice; 4, mouse parvovirus; 5, K virus; 6, lymphocytic choriomeningitis virus; 7, mouse polyoma virus grown in 3T6 cells; 8, cloned mouse polyoma virus from plasmid; 9, uninfected cell control; 10, no template control. The migration of molecular size markers is shown in the left lane of each gel.

Mouse polyoma virus inoculum and tissues recovered from infected BALB/c and nude mice were processed for analysis by use of the generic and mouse polyoma virus-specific PCR assays. Virus was detected in the inoculum by use of both PCR assays (data not shown). The mouse polyoma virus-specific and generic PCR assays amplified mouse polyoma virus DNA from multiple tissues collected from infected mice of both genotypes (Table 2), but not from tissues of sham-inoculated mice (data not shown). Virus was detected by use of the mouse polyoma virus-specific PCR assay from heart, mammary gland, and skin in most BALB/c mice at 2 weeks after inoculation, and only in the mammary gland and skin of one mouse at 4 weeks after inoculation. Similarly, in nude mice, virus was detected by use of the mouse polyoma virus-specific PCR assay in all tissues except kidney at 2 and 4 weeks after inoculation. The generic PCR assay was less sensitive than the mouse polyoma virus-specific PCR assay, as evidenced by amplification of no virus from BALB/c mouse tissues, and from fewer nude mice at most time points. The skin was the tissue that most frequently yielded positive PCR results from infected mice of either strain at either time point.

Table 2. Detection of mouse polyoma virus in tissues from experimentally infected mice, using the mouse polyoma virus specific and the generic (polyomavirus family) PCR assays

Mouse strain/date of tissue collection	Positive PV PCR results (No. positive/3 animals tested)									
	Heart		Liver		Kidney		Mammae		Skin	
	PV	PK	PV	PK	PV	PK	PV	PK	PV	PK
BALB/c - PIW 2	2	0	0	0	0	0	2	0	3	0
BALB/c - PIW 4	0	0	0	0	0	0	1	0	1	0
Nude - PIW 2	1	2	2	1	0	0	1	1	3	2
Nude - PIW 4	1	1	3	2	0	0	3	2	3	3

PV = Mouse polyoma virus-specific PCR assay.
PK= Polyomavirus family generic PCR assay (amplifies mouse polyoma virus and K virus).
PIW = Postinoculation week.

Discussion

We developed a series of PCR assays to screen biological specimens for the presence of mouse polyoma virus and K virus. The PCR assays specific for mouse polyoma virus and for K virus and a generic PCR assay for consensus regions of both viruses were designed. The mouse polyoma virus-specific PCR assay amplified only mouse polyoma virus, and was documented to be more sensitive than the generic PCR assay in amplifying DNA from cloned mouse polyoma virus or from mouse polyoma virus-infected cells. The K virus-specific PCR assay amplified only K virus DNA, and proved to be more sensitive than the generic PCR assay in amplifying K virus DNA. In addition, the mouse polyoma virus-specific and generic PCR assays were more sensitive than serologic testing in detecting mouse polyoma virus in infected mice.

The lesser sensitivity of the generic PCR assay may be explained in part by the use of inosines in the degenerate forward oligonucleotide primer to offset mismatches in sequences from mouse polyoma virus and K virus. Inosines anneal equally well to all nucleotides, but can affect the specific annealing of the oligonucleotide primer and increase nonspecific priming, thus lowering the efficiency of specific product amplification (18). Several generic primer sets were designed from the limited consensus regions of mouse polyoma virus and K virus, and the generic primer set used in this study provided the best sensitivity among those tested (data not shown). The generic primer set was designed to provide a single PCR assay to screen for the family of murine polyomaviruses; however, the lower sensitivity of the assay would limit the value of this assay for a "streamlined" approach for screening samples for murine polyomaviruses.

The BALB/c mouse infection study highlighted the sensitivity of PCR over antibody production assays in detecting mouse polyoma virus in a reagent or biological sample. The minimal antibody response of the BALB/c mice was likely the result of transient replication of virus in adult mice (12) in combination with use of a tissue culture-adapted strain of mouse polyoma virus. The strain of mouse polyoma virus was viable, as evidenced by the presence of virus in skin and mammary gland, tissues identified as sites of viral replication after primary viremia (12, 19, 20). The purpose of collecting tissues from infected mice for PCR analysis was not intended to mimic pathogenesis studies, but rather to confirm viability of the virus inoculum, and to evaluate the ability of these PCR assays to amplify virus from complex cellular specimens similar to tumors.

The nude mouse infection study documented the ability of the polyoma virus PCR to amplify viral DNA from multiple types of tissues. The tissue distribution of mouse polyoma virus in our study was comparable to that in other polyoma viral pathogenesis studies in adult mice (12, 20). The exploitable outcome of these experiments was the identification of skin as a site for polyoma virus replication in immunodeficient and immune competent mice. Skin from the flank was consistently positive for mouse polyoma viral DNA, and we subsequently tested skin from tail tips from these infected mice, and found viral DNA at this site as well (data not shown). Further studies should be performed to determine whether polyoma virus PCR analysis using tail snip as samples would be useful as a confirmatory test in mice, especially immune-compromised mice suspected of receiving mouse polyoma virus infected biomaterials.

These murine polyomavirus PCR assays we developed and validated have the potential to become important diagnostic tools to screen reagents and biological samples for the presence of contaminating mouse polyoma virus or K virus. These PCR assays will provide timely and accurate diagnosis of murine polyomaviral infections and will help prevent the inadvertent use of virus-contaminated reagents or animals in research.

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