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Original Research

Detection of Mouse Parvovirus in *Mus musculus* Gametes, Embryos, and Ovarian Tissues by Polymerase Chain Reaction Assay

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We used primary and nested polymerase chain reaction (PCR) assays to determine the presence of mouse parvovirus (MPV) in mouse sperm, oocytes, preimplantation embryos, and ovarian tissues collected from MPV-infected mice. The primary PCR assay detected MPV in 56% of the sperm samples. MPV was not eliminated by passing sperm samples through a Percoll gradient. After Percoll treatment, MPV was still present in 50% of the samples according to primary PCR assay. Oocyte samples that did not undergo extensive washing procedures had detectable MPV in 7% of the samples based on the primary PCR assay, but nested PCR assay detected higher (28%) infection rate. However, MPV was not detected in oocytes that underwent extensive washing procedures, as assessed by either primary or nested PCR assay. Although primary PCR did not detect MPV in embryos, a nested PCR assay determined that 50% of the embryos were positive for the virus. In addition, ovarian tissues were collected from 3 different mouse colonies with enzootic MPV infection. Ovarian tissue collected from 129CT, 101/R1, and Sencar mice had high incidence (38%, 63%, and 65%, respectively) of MPV infection on the basis of nested PCR amplification. These results demonstrate that mouse gametes, embryos, and ovarian tissues may be contaminated with MPV and therefore caution is necessary when infected germplasm is used for assisted reproductive technologies such as embryo transfer, establishing embryonic stem cell lines, in vitro fertilization, ovary transplantation, and intracytoplasmic sperm injection.

Abbreviations: ART, assisted reproductive technologies; MPV, mouse parvovirus; PCR, polymerase chain reaction; TL-HEPES, HEPES-buffered Tyrode lactate; ZP, zona pellucida

The past 2 decades have brought dramatic advances in the development of assisted reproductive technologies (ART), such as embryo transfer, in vitro fertilization, intracytoplasmic sperm injection, in vitro embryo culture, and ovarian tissue transplantation. Cryopreserved gametes (that is, sperm, oocytes) and tissues (that is, embryos, ovarian tissue) are an integral part of ART.[11,12] ART, in combination with cellular and genomic tools, have been used widely for the creation of genetically modified mice for studying gene functions and have helped the biomedical research community greatly. Cryopreservation of gametes and embryos is becoming the preferred method for maintaining large numbers of genetically modified mouse models. In addition, cryopreserved murine ovarian tissue with subsequent transplantation into immunoincompetent or genetically identical mouse recipients has proven to be a successful and cost-effective approach for the purpose of genome banking.[1,15,20] In fact, ovarian tissue cryopreservation is preferred for mouse lines in which male fertility is reduced or absent, such as the X–autosome translocation mouse models.[13]

Many infectious viral and bacterial agents can survive in commonly used cryoprotective agents (that is, glycerol, dimethyl sulfoxide) and can be transmitted through liquid nitrogen due to inappropriate storage conditions.[6] Vertical transmission of infectious agents such as hepatitis B and human immunodeficiency virus via gametes and embryos by ART during human infertility treatment are being addressed by the medical community.[10,21,23] In addition, importation of micromanipulated livestock embryos has been banned by many countries because these embryos have increased susceptibility to infectious agents due to loss of integrity of the zona pellucida (ZP), which functions as natural barrier.[40] With the broad use of mouse gamete and embryo manipulation and cryopreservation, similar disease transmission risks likely exist for the laboratory mouse.

Mouse parvovirus (MPV) is one of the most prevalent infectious agents in laboratory mice.[19,24] MPV infection is clinically asymptomatic in both adult and newborn mice.[18] However, the use of infected research mice may present serious confounding effects and thus has the potential to compromise the interpretation of research findings.[25] MPV seroconversion takes place between 1 and 2 wk after initial exposure to the virus, and several methods have been developed and used for diagnosis of MPV, including enzyme-linked immunoabsorbent assays, indirect fluorescent antibody assays, and hemagglutination inhibition assays.[2,18,24,33] Newer molecular diagnostic assays such polymerase chain reaction (PCR) assays and the fluorogenic nuclease PCR method also have been used to detect MPV.[32] These molecular diagnostic as-

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says can be used to directly test for the presence of MPV in biologic materials such as cell lines, tissues, gametes, and embryos.[3,8]

In this study, we used primary and nested PCR assays to determine whether MPV was present in gametes, embryos, and ovarian tissues collected from MPV-infected mice. We also tested whether common practices, such as Percoll gradient separation of sperm and washing of oocytes and embryos, could be used to eliminate MPV from these biomaterials.

Materials and Methods

Animals. All animal studies were performed in accordance with guidelines of the University of Missouri Animal Care and Use Committee and the *ILAR Guide for the Care and Use of Laboratory Animals*.[28] We obtained 4- to 12-wk-old outbred Sencar mice from a colony enzootically infected with mouse parvovirus, murine hepatitis virus, Sendai virus and Theiler murine encephalomyelitis virus as determined by repeated serologic testing. The colony was infested with *Syphacia obvelata* as evidenced by positive perianal tape testing and microscopic examination of cecal contents. *Pasteurella pneumotropica* and multiple *Helicobacter* species (including *H. bilis*, *H. rodentium*, and *H. typhlonius*) also were enzootic in this colony. On arrival at the University of Missouri, mice were housed in isolator caging at 20 to 25 °C in a controlled lighting environment (10 h dark, 14 h light) and provided free access to water and standard pelleted rodent chow. Animals were monitored daily by the animal care staff, and no signs of clinical illness or disease were noted in any of the mice during the entire study. MPV- negative (ICR control) mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) at 6 to 10 wk of age and subsequently used to collect oocytes, sperm samples, embryos, and ovarian tissues for use as negative controls during these studies. All donor mice were euthanized by inhaled CO_2 overdose followed by cervical dislocation, and mesenteric lymph nodes, oocyte, embryo, ovarian tissues, and sperm were collected. MPV PCR was performed on mesenteric lymph nodes to confirm that donors were infected with MPV; mice not infected with MPV were excluded from the study.

Sperm collection and Percoll separation. The cauda epididymides of 4- to 12-wk-old Sencar mice were excised bilaterally and placed on a sterile filter paper to remove adherent blood and fat tissue and then were cut and placed in microfuge tubes each containing 500 µl Dulbecco phosphate-buffered saline to release spermatozoa. Samples were allowed to incubate for 10 min at 37 °C to allow the sperm to swim out of the epididymis and into the medium. The epididymal tissue then was removed manually with fine forceps from the microfuge tube, and the medium containing the spermatozoa was either frozen immediately for subsequent MPV testing or used for Percoll separation experiments. Sperm samples used for Percoll experiments were mixed gently for 5 min, layered on a discontinuous 90%:45% Percoll gradient in a ratio of 1:1:1 ml (cell sample: 45% Percoll:90% Percoll), and centrifuged at 500 × *g* for 15 min to remove blood cells, immotile sperm, and epithelial cells. The cells separated into 3 distinct layers. The top 2 layers (containing debris, nonsperm cells, and immotile cells) were discarded, and the bottom pellet (containing motile spermatozoa) was washed with modified S-PBS solution[41] and then centrifuged at 400 × *g* for 10 min. The supernatant was removed, leaving approximately 100 µl of cells. The sperm samples were washed with S-PBS twice after Percoll gradient separation, resuspended in 20 µl S-PBS in a microfuge tube, and frozen for subsequent MPV testing.

Oocyte and embryo collection. Four- to 12-wk-old female mice were superovulated by intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin (Sioux Biomedical, Sioux City, IA or Sigma Chemical, St Louis, MO) followed 48 h later by intraperitoneal injection of 7.5 IU human chorionic gonadotropin (Sigma). These mice then either were euthanized approximately 16 h after injection of human chorionic gonadotropin (for collection of oocytes) or were placed with male mice immediately after injection and then were euthanized 60 to 72 h after mating (for collection of morula-stage embryos). For oocyte collections, oviducts containing cumulus oocytes complexes were removed and washed twice in HEPES-buffered Tyrode lactate (TL-HEPES) solution[4] supplemented with 0.3% bovine serum albumin. Each oviduct then was torn gently to release the complexes into fresh TL-HEPES solution. Cumulus cells were removed from the oocytes by treating them with 1 mg/ml hyaluronidase in TL-HEPES. Oocytes then were collected by use of a pulled glass pipette (inner diameter, 100 to 120 µm) and transferred successively into dishes containing fresh TL-HEPES solution for 3 washes (standard washing) or 6 washes (extensive washing). Oocytes from each female mouse were handled separately and were placed into a microfuge tube containing 10 µl of phosphate-buffered saline solution for subsequent MPV PCR testing.

For collection of embryos, the uterus and oviducts were removed and placed in TL-HEPES solution and were washed twice in fresh TL-HEPES prior to recovery of the embryos. Embryos were flushed from the reproductive tract into TL-HEPES and then were transferred successively into dishes containing fresh TL-HEPES for 6 washes. Embryos from each female mouse were handled separately, and all embryos collected from a single female mouse were transferred into a microfuge tube containing 10 µl of Dulbecco phosphate-buffered saline solution for subsequent MPV PCR testing.

Ovarian tissue collection. Female donors obtained from enzootically MPV-infected mouse colonies were euthanized, and both ovaries were removed, separated from associated connective tissues (that is, ovarian bursa and fat tissue), and washed 3 times with TL-HEPES solution supplemented with 3 mg/ml bovine serum albumin, 0.2 mM sodium pyruvate, and 50 mg/ml gentamycin sulfate. Mice from the X-linked translocation mutant stock 129CT and the inbred strain 101/R1 were obtained from a different facility (Oak Ridge National Laboratory, Oak Ridge, TN) than the outbred 4- to 12-wk-old Sencar mice. Outbred ICR mice were purchased from Harlan Sprague Dawley and served as MPV-negative control mice.

Primary and nested PCR assays. For PCR assays, total DNA was extracted using DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's guidelines for tissue extraction with the following minor modifications. A total of 10 to 12 embryos or oocytes from a single donor and both ovaries form a single donor and 25 mg of ovarian tissue from a single donor were used for DNA extractions. For embryo and oocyte samples, 2 µg of carrier poly(A) RNA was added to the sample after the initial digestion step with proteinase K. For all samples, both wash steps were repeated once, and the final elution volume was 200 µl. PCR was performed using primers specific for the MPV VP2 protein.[5] Each PCR reaction used 1 unit of *Taq* polymerase (Roche, Indianapolis, IN), 200 µM each dNTP, 1 µM of each primer, 1.5 mM $MgCl_2$, and 5 µl of DNA solution. PCR reactions were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA). An initial denaturation step at 94 °C for 30 s was

Table 1. MPV infection of sperm before and after Percoll treatment

Treatment	Donor strain	Donor age (wk)	No. of donors	No. positive by primary PCR (%)
Swim-up*	Sencar	4–6	20	5 (25)
Swim-up	Sencar	10	25	14 (56)
Percoll separation	Sencar	10	8	4 (50)
Swim-up	Sencar	10–12	12	6 (50)
Swim-up	ICR (control)	8–10	12	0 (0)

*Supernatant of the solution in the test tube containing mostly live sperm.

Table 2. MPV infection of oocytes after standard (3 washes) or extensive (6 washes) washing

Treatment	Donor strain	Donor age (wk)	No. of donors	No. positive by primary PCR (%)	No. positive by nested PCR (%)
Standard washing	Sencar	4–6	9	1 (11)	3 (33)
Standard washing	Sencar	10–12	20	1 (5)	5 (25)
Extensive washing	Sencar	10–12	15	0 (0)	0 (0)
Standard washing	ICR (control)	6–8	15	0 (0)	0 (0)

performed, followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 10 s, and extension at 72 °C for 30 s. A final extension at 72 °C for 7 min was performed.

For nested PCR, the first amplification reaction used primers in the VP2 region (3552F, 3' CCA TTG AGA ACA CTC AAC G 5'; 4154R, 3' AGC AGT TAG TGG TCC ATA GC 5'), and the second reaction used a previously published set of internal primers for MPV VP2.[5] Cycle conditions remained the same as described, except that only 35 cycles were performed for each reaction and 1 µl of the initial PCR reaction was used as template in the secondary PCR reaction. All PCR experiments contained negative (no DNA) and positive (MPV viral DNA) control reactions in addition to the test samples. Amplicons underwent 2% agarose gel electrophoresis, ethidium bromide staining, and visualization under ultraviolet light. All negative controls were uniformly negative, and all positive controls were uniformly positive.

Results

Detection of MPV in sperm. The incidence of MPV in sperm samples is summarized in Table 1. According to primary PCR testing, 5 of the 20 (25%) samples from 4- to 6-wk-old mice were positive for MPV, whereas 6 of the 12 (50%) collected from 10- to 12-wk-old mice tested positive. In a separate experiment, sperm samples collected from 10-wk-old male mice were separated on Percoll density gradients in an effort to eliminate MPV. Without Percoll treatment, 14 of 25 (56%) of sperm samples were positive by primary PCR assay; with Percoll gradient separation, MPV was detected in 4 of 8 (50%) of the sperm samples. However, none of the 12 sperm samples collected from the control group of ICR mice was positive for MPV by primary PCR assay.

Detection of MPV in oocytes. The incidence of MPV in oocyte samples is summarized in Table 2. The incidence of MPV was lower for oocytes than sperm samples. The incidence of MPV in oocyte samples collected from 4- to 6-wk-old mice and subjected to a standard washing procedure was 11% (1 of 9 samples) when we used the primary PCR assay but 33% (3 of 9 samples) after nested PCR. In a separate experiment, we tested oocyte samples collected from 10- to 12-wk-old mice for MPV after either standard washing or extensive washing. The oocytes collected and subjected to standard washing (3 washes) had a similar incidence of MPV (5% positive after primary PCR, 25% positive with nested PCR) as seen for 4- to 6-wk-old mice. However, none of the 15 oocyte samples that were subjected to an extensive washing procedure (6 washes) were positive for MPV infection in either primary or nested PCR assays. None of the 15 oocyte samples collected from ICR mice (control) was positive for MPV by either primary or nested PCR assay.

Detection of MPV in embryos. The incidence of MPV infection in embryos is summarized in Table 3. Primary PCR assays did not detect MPV in any of the embryo samples tested. However, nested PCR showed that 50% of the embryo samples from both 4- to 6-wk-old (2 of 4) and 6- to 8-wk-old (5 of 10) mice were positive for MPV. In contrast, nested PCR assay tests were negative for all 12 embryo samples collected from 10- to 12-wk-old mice. None of the 14 embryos collected from control ICR donors was positive for MPV.

Detection of MPV in ovarian tissue. The incidence of MPV infection in ovarian tissues is summarized in Table 4. None of the ICR control mice was positive for MPV by either primary or nested PCR. Although none of the ovarian tissue samples recovered from 129CT mice had detectable viral DNA according to primary PCR assays, nested PCR revealed a 38% positive rate. Primary PCR revealed MPV-positive ovaries in 101/R1 (21%) and Sencar (35%) mice; with nested PCR, rates were 63% for 101/R1 mice and 65% for Sencar mice.

Discussion

Rederivation by embryo transfer has become the primary mechanism to eradicate MPV from enzootically infected colonies.[9,26,37] In addition, semen and embryo cryopreservation techniques have enabled storage of important mouse models, so that models no longer need to be kept as live colonies when they are not in use.[11,12,27,31] Cryopreserved or freshly collected sperm can be used in combination with oocytes for in vitro fertilization or intracytoplasmic sperm injection to generate embryos, which can then be transferred to recipient female mice to generate live pups or cryopreserved for storage and later recovery of live animals. However, the transfer or use of embryos or gametes between institutions requires consideration of the risk of disease transmission. Therefore, although existing reproductive technologies are an important colony management tool, the use of embryos, sperm, or oocytes collected from donor animals with contagious diseases may serve as a potential source for the introduction of disease into the animal facility. In this study, we found MPV present in

Table 3. MPV infection of preimplantation embryos after extensive washing (6 washes)

Donor strain	Donor age (wk)	No. of donors	No. positive by primary PCR (%)	No. positive by nested PCR (%)
Sencar	4–6	4	0 (0)	2 (50)
Sencar	6–8	10	0 (0)	5 (50)
Sencar	10–12	12	0 (0)	0 (0)
ICR (control)	6–8	14	0 (0)	0 (0)

Table 4. Summary of incidence of MPV in ovarian tissues collected from 101/R1, 129CT, Sencar, and ICR mice

Donor strain	Donor age (wk)	No. of donors	No. positive by primary PCR (%)	No. positive by nested PCR (%)
101/R1	10–12	19	4 (21)	12 (63)
129CT	10–12	8	0 (0)	3 (38)
Sencar	4–12	81	28 (35)	53 (65)
ICR (control)	6–8	14	0 (0)	0 (0)

embryos, sperm, and oocytes. Our findings highlight the need for disease surveillance when working with embryos, sperm, and oocytes that have been obtained from a colony with a history of MPV infection or with an unknown history of infectious disease.

Contaminated sperm samples may present an increased risk of infectious disease, particularly when used in ART. Although intact cells may naturally be protected from infectious agents, damage to the plasma membrane or ZP of oocytes during ART procedures may provide opportunities for the entry of pathogens. For example, during intracytoplasmic sperm injection, both the ZP and oolemma must be penetrated to deposit spermatozoa into the ooplasm.[29] Perforation of the oolemma with the microinjection pipette impairs the oocyte's natural ability to prevent introduction during fertilization of infectious agents that are attached to sperm plasma membrane.

Disease agents such as bacterial and fungal pathogens are too large to cross the ZP or sperm plasma membrane, and even if they do adhere to it, standard antibiotics (that is, penicillin, streptomycin) in the washing medium can be used to inactivate them.[35] Therefore, viral agents are the primary disease concern when working with sperm, oocytes and embryos. Gamete-mediated infection is not generally considered a noteworthy contributor of embryonic infection, because most of the infectious agents that have been found in semen are actually in the seminal fluid or its concomitant cells and are not associated with spermatozoa.[6,14] However, each viral pathogen must be investigated to determine its ability to adhere to the sperm plasma membrane and, if adherent, whether the virus can be removed by cell separation techniques. Several methods, such as 'swim-up' protocols and Percoll and Ficoll gradients, have been used to isolate spermatozoa that are free of infectious agents for further reproductive use.[16,17,30] However, the data in the present study clearly show that MPV remain even after Percoll separation, suggesting firm adherence of MPV in or on spermatozoa.

Prevention of viral transmission is highly dependent on the location of the virus (that is, whether in cells or adherent to the cell), how strongly viruses adhere to the ZP, and whether the ZP is intact. These factors contribute markedly to the success of virus elimination efforts according to conventional treatment methods. For example, washing and trypsin treatment procedures to remove viruses adhering to the ZP have been used frequently with murine embryos.[7] Although proper washing and trypsinization are effective for removing very high levels of viral particles from oocytes and embryos[7], this approach may not eliminate all viral agents from oocytes and embryos. For example, Lavilla and colleagues[22] reported that standard washing and trypsin treatment did not eliminate Sendai virus adhering to the ZP of mouse embryos. In addition, viral agents residing within embryos would be completely resistant to standard washing and trypsin treatment. Huang and colleagues[17] recently investigated potential transmission of hepatitis B virus into mouse oocytes by coincubating metaphase II mouse oocytes with viral plasmid DNA. Their study revealed that hepatitis B viral DNA could pass through the ZP and oolemma and integrate into embryonic genome. Our findings demonstrate that extensive washing of oocytes reduces the MPV infection to undetectable levels on PCR testing. This suggests that MPV particles are loosely adherent to the ZP of oocytes. In contrast, embryos that we washed extensively were positive by nested PCR, indicating that washing alone is insufficient to remove MPV from embryos.

Cryopreservation and subsequent transplantation of ovarian tissue is a useful reproductive technique that has been used to rescue transgenic strains with reproductive problems.[1,36,38,39] However, tissues that are infected with infectious agents carry a considerable risk of disease transmission to the recipient and to offspring that are derived from infected gonads. In the present study we found that ovarian tissue collected from mice enzootically infected with MPV contains viral DNA, raising the question of whether offspring derived from ovarian tissue transplantation are infected with MPV. A few studies have examined whether ovarian tissue containing murine pathogens can transmit the pathogen to recipient female mice or their offspring. Scavizzi and Raspa[34] demonstrated that ovarian tissue recovered from mice infected with mouse hepatitis virus was positive for the virus by reverse transcriptase-PCR. In addition, transplanted ovarian tissue collected from donors infected with mouse hepatitis virus resulted in disease transmission to the recipient females and their offspring. In another study,[35] these authors evaluated ovarian tissue for the presence of *H. typhlonius* and found that although ovarian tissue contained this agent, it was not transmitted via ovarian tissue transplantation to the recipient females and their offspring.[35] These results demonstrate that each viral agent must be assessed for its presence in ovarian tissue and its ability to transmit disease when transplanted to recipient female mice.

The detection of MPV in mouse sperm, oocyte, embryos, or ovarian tissues by PCR does not necessarily imply the presence of infective virions and the possibility of transmission through ART. Therefore, although we can confirm with the certainty that viral

DNA is present, we cannot confirm whether this state is sufficient to lead to infection in the offspring. However, our current data clearly suggest the importance of performing indepth investigation to elucidate these possibilities. These data further suggest the importance of performing mandatory testing on offspring that are reconstituted by using ART from donor animals that are infected with MPV. Future experiments will be conducted to test the hypothesis that MPV-infected gametes, embryos, and ovarian tissue can produce MPV-positive fetuses.

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