MICROBIAL PATHOGEN CONTAMINATION IN MOUSE

GAMETES AND EMBRYOS

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

MICROBIAL PATHOGEN CONTAMINATION IN
MOUSE GAMETES AND EMBRYOS

presented by Lin Zhang,

a candidate for the degree of master of science,

and hereby certify that, in their opinion, it is worthy of acceptance.

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ABSTRACT

In recent years, large numbers of transgenic and knockout mice have been generated and it is anticipated that the numbers of lines will continue to increase as scientists further investigate the genome and gene functions. Cryopreservation provides an efficient and effective way to maintain these unique genetically modified rodent lines. However, there is a great risk of transmitting various diseases via the cryopreserved gametes and embryos. Microbial pathogens may come from both male donors (semen) and female donors (oocytes and embryos). Although pathogens associated with gametes and embryos of cattle and swine have been evaluated to a great extent, very little research has been conducted on microorganisms in mouse germ cells. Our hypothesis is that microbial pathogens may associate with mouse gametes and embryos. To investigate which of the commonly found murine pathogens contaminate mouse gametes and embryos, seven commonly found pathogens in research mice were evaluated in semen, oocyte-cumulus complexes and embryos collected from naturally infected mice in the first part of my study. These seven pathogens are: mouse parvovirus, *Helicobacter, Mycoplasma pulmonis, Pasteurella pneumotropica,* murine norovirus, mouse hepatitis virus and Theiler's murine encephalomyelitis virus. Five pathogens, mouse parvovirus, *Helicobacter,* mouse hepatitis virus, murine norovirus, Theiler's encephalomyelitis virus, were found in mouse semen, and mouse parvovirus, *Helicobacter,* mouse hepatitis virus, murine norovirus were found in mouse oocytes and embryos. These findings indicate there is potential risk of transmitting microbial pathogens via cryopreserved gametes and

embryos.

Recent advances in the methodologies for cryopreservation of mouse spermatozoa have opened up a number of opportunities for storage of transgenic mouse strains and rederivation by assisted reproductive technologies (ART) such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo transfer, ovarian tissue transplantation. Mouse parvovirus is one of the prevalent pathogens in research animals and mouse gametes and embryos as well. Therefore, the second part of this study focused on evaluating mouse parvovirus and determining the effect of infection in male mice on *in vitro* fertilization including how it will change fertilization rate, cleavage rate and embryonic development and whether Mouse parvovirus was transmitted to embryos at blastocyst stage. Our hypothesis is Moue parvovirus carried by sperm transmits to blastocysts and affects fertilization, cleavage and embryonic development. In this study, *in vitro* fertilization was performed with sperm collected from ICR mice that were inoculated with Mouse parvovirus-1 and oocytes collected from ICR uninfected mice. In control group, IVF was performed by using sperm and oocyte collected from ICR uninfected mice. We did not find mouse parvovirus-1 infection in male mice transmission to *in vitro* produced embryos and there was no effect on fertilization rate, cleavage rate and embryonic development during *in vitro* fertilization.

CHAPTER 1 MICROBIAL PATHOGENS IN MOUSE GAMETE AND EMBRYO

I. Introduction and background

1. General information of cryopreservation of gametes and embryos

Advances in genetic manipulation have resulted in a dramatic increase in the number of transgenic mutant mouse lines (Simpson *et al.*, 1997). Maintenance of these numerous mutant strains as live animals is not feasible due to limited resources. Cryopreservation of the animal germline has shown to be an efficient method to deal with this challenge (Mobraaten, 1999; Glenister and Thornton, 2000; Critser and Mobraaten, 2000).

Cryopreservation maintains the biological material at low temperatures of -196°C, in liquid nitrogen, and the viable material can be recovered for later usage. The most regularly cryopreserved cells are the germ cells including spermatozoa, oocytes, embryos and the reproductive tissues such as the testicular tissue and the ovarian tissue (Woods *et al.,* 2004). Successful cryopreservation of mouse embryos was reported independently by Whittingham *et al.* (1972) and Wilmut (1972). In 1977, Whittingham published a successful protocol for the cryopreservation of unfertilized mouse oocytes. Cryopreservation of mouse ovarian tissue and recovery of fertile mice following the grafting of frozen/thawed tissue had first been reported by Parrott in 1958 and 1960. Polge *et al.* (1949) first described a reproductible method to freeze human

and avian spermatozoa. Indeed, pregnancies and live-born offspring from cattle fertilized with frozen/thawed semen were reported in 1952 (Polge and Lovelock 1952; Polge and Rowson 1952). Unfortunately, mouse spermatozoa proved far more difficult to cryopreserve, and it was only during the 1990s that apparently successful methods appeared in the literature (Tada *et al.* 1990; Okuyama *et al.* 1990; Takeshima *et al.* 1991; Nakagata 1994; Songsasen *et al.* 1997; Sztein *et al.* 1997; Marschall *et al.* 1999; Thornton *et al.* 1999).

Cryopreservation has been shown to be an effective method to keep transgenic animal lines. These animals can be brought back by using assisted reproductive technologies (ART) including *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo transfer and ovarian tissue transplantation. As a popular animal model, mice are widely used for studying gene functions and certain human diseases. Cryopreservation of mouse sperm and embryos has achieved great success. However, compared to other mammalian species, embryo-freezing protocols used for mice have yielded very little success for cryopreservation of oocyte (Woods *et al.*, 2004). In addition to some problems in cryopreservation technology, there is a major concern on the potential for disease transmission via frozen stocks since many of the current transgenic and knockout strains are infected with pathogens.

2. General information of microbial pathogens in laboratory animals

There is abundant evidence documenting that infection of laboratory animals can compromise and invalidate animal-based researches (Baker, 1998, 2003; Lipman

and Perkins, 2002; Nicklas *et al*., 1999; NRC, 1991). Thus, it is critical that laboratory animals used in research studies be free of microbial pathogens. Although significant strides such as health monitoring (Baker 1998) have been made in reducing microbial pathogens in laboratory animals, surveys of research institutions in the U.S. indicate that a high percentage of institutions maintain and utilize mice and rats harboring microbial pathogens (Jacoby and Lindsey, 1997; Riley, 1999) , as shown in Table 1-1.

Table 1-1 Prevalence of microbial pathogens in mice and rats

Microbial pathogen [a]	Prevalence of institutions with infected mice (%)	Prevalence of institutions with infected rats (%)
Parvovirus	40	28
Coronavirus	72	9
Theiler's murine encephalitis virus (TMEV)	35	7.5
Rotavirus	28	3
Helicobacter [b]	30	18
Pneumonia virus of mice (PVM)	22	9
Sendai virus	21	2
Reovirus (Reo3)	15	3
Mycoplasma	18	12
Adenovirus	7.5	2
Lymphocytic choriomeningitis virus (LCMV)	4	3

[a] *Selected pathogens. Data based on survey conducted by Jacoby and Lindsey (1997) and Riley et al. (1999).*

[b] *Believed to be an underestimate since only a limited number of institutions test for helicobacters.*

Under some circumstances, the risk of microbial pathogen infection is significantly greater in some laboratory transgenic mouse populations than in wild-type stocks and strains. For example mice with mutations that result in deficiencies in T cells, T and B cells (severe combined immunodeficiency mice) are notoriously susceptible to infections by the ubiquitous opportunistic fungus *Pneumocystis carinii* (Keely *et al.*, 2004), opportunistic bacteria such as the *Pasteurellas* and *Staphylococci* and viral pathogens such as Mouse hepatitis virus. Some mutant mouse strains are more susceptible to infections due to abnormal immune response. For example, SJL mice that have deficiency in natural killer cells, AKR, DBA/2, and SWR mice that have complement component 5 deficiencies, and toll-like receptor 4 deficiencies in C3H/HeJ mice may be susceptible to some infectious diseases (Barthold, 2002; Poltorak *et al*., 1998; Franklin, 2006). Realizing the unique health issues associated with some genetically manipulated rodents, it is not surprising that an increase in infections and diseases in laboratory mice and rats has been noted since the advent of genetically-manipulated mice and rats (Riley, 1999).

While cryopreservation has been used for storage of rodent stocks and strains in recent years, its use will greatly expand in the upcoming years as the number of transgenic and knockout rodent strains created continues to increase exponentially, requiring large-scale cryopreservation to prevent loss of valuable genetically manipulated rodent lines. Efforts to cryopreserve genetically-manipulated rodents must be linked to efforts to prevent transmission of microbial pathogens to offspring.

If not, extensive efforts in cryopreservation will have limited value since the end product will be compromised by the deleterious impact of microbial pathogens on progeny.

3. General information about microbial pathogen contamination in cryopreserved gamets and embryos

The microbial pathogens may potentially originate from the male donors (semen) or the female donors (oocytes and embryos) (Tallec, 2001a). In livestock and humans, a large number of studies have been conducted examining pathogen contamination in sperm, gametes and embryos (Stringfellow and Givens, 2000; Perry 2007; Pudney, 1999; Wrathall, 2006). While findings in livestock and humans may not apply to what will be found in rodents, the fact that pathogen contamination of sperm and embryos has been found in multiple species (cattle, sheep, swine and humans) strongly suggests that similar issues exist in mice.

Semen contamination:

Semen consists of spermatozoa, seminal fluid and non-sperm cells such as epithelial cells, lymphocytes, leukocytes and macrophages. Each component could be a potential vehicle for the transport of microbial pathogens. Systemic and local infections of the reproductive tract, as well as the inadvertent introduction of microorganisms during semen processing represent possible routes of pathogen contamination of semen (Bielanski, 2007).

In humans and livestock, contamination of semen has been extensively documented. In humans, human immunodeficiency virus (Baccetti *et al.,* 1994; Quayle *et al.,* 1997; Quayle *et al.,* 1998; Pudney *et al.,* 1999), hepatitis B virus (Hadchouel *et al.*, 1985; Huang *et al.*, 2003) and human-herpesvirus-8 (Bagasra, 2005) have been reported to be associated with semen. In cattle, bovine herpesvirus-1 (Van Oirschot, 1995; Elazhary *et al.*, 1980; Van Engelenburg *et al.,* 1993), bovine viral diarrhea virus and bluetongue virus (Wrathall, 2006) are found associated with semen. In pigs, porcine reproductive and respiratory syndrome virus (Sur, 1997), porcine circovirus II (Kim *et al.*, 2001; Larochelle *et al.*, 2000; Tallec *et al.,* 2001b), and porcine parvovirus (Johnson *et al.,* 1976) are also found in semen.

Mouse parvovirus is found in mouse sperm and could not be eliminated by a Percoll gradient centrifugation (Agca *et al.,* 2007). Microbial contamination of semen has been reported to cause infection of female recipient (Meyling and Jensen, 1988) and reduce fertilization and embryonic cleavage rates (Guerin *et al.*, 1992).

Oocyte and embryo contamination:

Oocytes and embryos are surrounded by the zona pellucida, a natural barrier protecting them from microbial pathogens. In livestock species, especially in bovine, extensive research has also been documented that bacterial and viral pathogens can be transmitted to oocytes and embryos (Perry, 2007; Stringfellow and Givens, 2000; Vanroose *et al.,* 1999). Microbial contamination in embryos derived *in vivo* is different from embryos derived *in vitro* (Tallec *et al.*, 2001a). In one study, embryos

from artificially or naturally infected donor cows were evaluated for the infectious status. After washing or washing with trypsin according to the Manual of IETS (Stringfellow and Seidel,1998), embryos collected from cows artificially or naturally infected by bovine leukemia virus, bovine viral diarrhea virus, bluetongue virus, foot-and-mouth disease, bovine herpesvirus-1, rinderpest virus, *Brucella abortus* and *Chlamydia psittaci* were all negative for infected agents (Stringfellow and Givens, 2000). Thus, pathogens were not associated with washed or trypsin washed embryos even when *in vivo* exposure to pathogens was confirmed by finding infectious agent in the uterine recovery medium. The results obtained for *in vivo* produced embryo cannot be extrapolated to *in vitro* produced embryo because they are different in morphology and physiology (Wright and Ellington, 1995), which may change host-pathogen interactions and allow increased pathogen transmission. IVF and ICSI circumvent the normal host defense system, thus pathogens that would have been eliminated by the host may proliferate and/or survive the ART process (Kim, 1998). For example, bovine herpesvirus-1 (BHV-1) and bovine viral diarrhea virus (BVDV) have been at the center of consideration because they are ever present in cattle populations and are known to occur in serum and reproductive tissue (Baker, 1995; Brock *et al.,* 1998; Rossi *et al*., 1980). These two viruses are found in oocyte-cumulus complex from acutely infected cattle (Bielanski and Dubuc, 1994, 1995; Bielanski *et al*., 1998). They are also found in the *in vitro*-derived bovine embryos (Vanroose *et al*., 1999). Embryo processing (washing and trypsin treatment) are ineffective for removing them (Bielanski and Bubuc, 1993; Trachte *et al.,* 1998). Furthermore,

embryo treatments are equally ineffective for removal of bluetongue virus (Langston *et al.,* 1999), foot-and-mouth disease virus (Marquant-Le Guienne *et al.*, 1998) and *leptospira* (Bielanski and Surujiballi, 1996) from *in vitro* produced embryos after in vitroartificial exposure.

Extensive research has documented that bacterial and viral pathogens can be transmitted to oocytes and embryos. Few studies regarding microbial pathogen contamination in rat and mouse oocytes and embryos have been published. In the few studies that have been performed it has been shown that bacterial and viral pathogens can be introduced into oocytes via contaminated spermatozoa (Ericsson *et al*., 1971) and can pass through the ZP in early mouse embryos (Gwatkin, 1967; Wu and Meyer, 1972). For example, in mice infected with lymphocytic choriomeningitis virus, virus has been detected inside the ZP-intact ova from mice that are persistently infected (Mims, 1966). In contrast, Carthew and colleagues (1985) showed that mouse hepatitis virus (MHV) does not infect ZP-intact embryos. Interestingly, while MHV did not infect the embryos in the experiments described by Carthew *et al.* (1985), the foster mother became acutely infected, and was capable of shedding MHV, transmitting the virus to susceptible mice and causing a major outbreak of disease. Regardless of the mechanism, it is clear that contaminated oocytes and embryos can transmit microbial pathogens and potentially cause outbreaks of disease. In one study, mouse parvovirus is found in oocytes not subjected to extensive washing. However mouse parvovirus is not detected in oocytes following extensively washed (Agca *et al*., 2007).

4. General information about seven pathogens investigated in this study

In this study, we investigated whether mouse semen, oocyte-cumulus complex and embryos carry pathogens or not. There are many microbial pathogens that can infect laboratory mice. In this study, we evaluated the presence of seven commonly found pathogens in research mice that are also naturally found in Sencar mouse. Sencar mouse is derived from crossing Charlie's river CD-1 and skin tumor sensitive mouse. This colony is very sensitive to skin tumor induction. Previous data in our lab showed that colony was infected by several apthogens and these pathogens will be investigated in this study including seven pathogens: mouse parvovirus (MPV), *Helicobacter sp.*, *Pasteurella pneumotropica, Mycoplasma pulmonis,* murine norovirus (MNV), mouse hepatitis virus (MHV) and Theiler's murine encephalomyelitis virus (TMEV). These seven pathogens either cause diseases or subclinical symptoms in laboratory mice (Baker, 1998). If gametes and embryos collected from the infected mice carry these pathogens, there is a significant potential risk for cryopreservation and assisted reproductive technology. Microbial contamination of spermatozoa has been reported to reduce fertilization and embryonic cleavage rates (Guerin, 1992). According to Guerin, bovine viral diarrhea virus contamination in bull semen has an important effect on very early embryonic mortality. It could cause problems for storage of transgenic mutant animal lines if contamination in gametes affects the normal development of embryos as BVD. In addition, carrying pathogens in gametes and embryos has significant risk of transmitting pathogens to recipients and pups. Microbial contamination of sperm has

been reported to cause infection of female recipient (Meyling and Jensen, 1988).

Seven pathogens investigated in the first part of this study affect research trials in different ways. Mouse parvovirus can have deleterious effects on research trials due to *in vitro* and *in vivo* immunomodulatory effects, tumor suppression and contamination of cell cultures and tissues originating from mice (McKisic *et al.,* 1993; 1996, 1998). *H. hepaticus* infects the liver and intestinal tract and causes enterocolitis, typhlitis, and hepatitis in germ free mice (Fox *et al.,* 1996a). Furthermore, in susceptible strains (e.g., A/JCr mice), *H. hepaticus* causes chronic hepatitis and hepatocellular carcinoma (Fox *et al.*, 1996b, Ward *et al.*, 1994). *H. bilis* colonizing the liver and intestinal tract of mice, has been associated with multifocal chronic hepatitis, and in particular, induces inflammatory bowel disease in interleukin-10-deficient (IL-10$^{-/-}$) mice (Burich *et al.*, 2001, Fox *et al.*, 1995). *H. typhlonius* causes colitis and typhlitis in severe combined immunodeficient (SCID) and IL-10$^{-/-}$ mice (Franklin *et al.*, 1999). Clinical diseases reportedly caused by *P. pneumotropica* are ophthalmitis, conjunctivitis, and mastitis, but reported generally limited to lesions of the skin and adnexal structures (National Research Council, 1991). *M. pulmonis* may disseminate widely throughout the host and therefore may alter the experimental results in numerous ways. The effects thus far reported include (1) alteration of pulmonary carcinogen and immune responses, ciliary function, and cell kinetics; (2) reproductive efficiency; (3) adjuvant- and collagen-induced arthritis; and (4) systemic immune responses (Faulkner *et al.,* 1995; National Research Council, 1991; Ross *et al.*, 1992). Mouse hepatitis virus is highly contagious and causes a wide

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spectrum of disease manifestations ranging from subclinical infections to high mortality, depending on the virus strain and host age, genotype or immune status (Barthold & Smith 1989; Barthold *et al.* 1993). There is no direct evidence that subclinical murine norovirus (MNV) infection causes any interference with research. However, the virus replicates in cells of the macrophage line, and host resistance to MNV apparently requires activity of interferon-dependent systems (Chang *et al*., 2006). These observations leave open the possibility that MNV could impact research into macrophage function or interferon-dependent pathways. Natural infection of Theiler's murine encephalomyelitis virus (TMEV) in mice has reportedly interfered with the study of other viral infections (National Research Council, 1991). In addition, TMEV slows the conduction of spinal motor and somatosensory evoked potentials (Iuliano *et al*., 1994) and could compromise studies involving the CNS.

It is very important to study microbial pathogens in mouse gametes and embryos and develop efficient methods to avoid and eliminate pathogens. Research will benefit greatly from the use of pathogen-free animals.

II. Microbial pathogens in mouse semen

1. 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. Sencar mouse and ICR mouse were ordered from Harlan (Indianapolis, IN). Sencar mouse is derived from crossing Charlie's river CD-1 and skin tumor sensitive mouse. This colony is very sensitive to skin tumor induction. The Sencar mouse colony used in these studies has been documented to be naturally infected by several pathogens including mouse parvovirus, *Helicobacter sp., Mycoplasma pulmonis* and *Pasteurella pneumotropica,* mouse hepatitis virus, murine norovirus and Theiler's murine encephalomyelitis virus (TMEV). The presence of these infectious agents was confirmed by the serology and PCR tests. The ICR mouse colony used in this study was routinely subject to health monitoring by the Research Animal Diagnosis Lab (RADIL) at the University of Missouri-Columbia. Results confirmed that this strain was free of common murine microbial pathogens including the seven pathogens to be investigated in this study. Mice were housed in microisolator caging in a temperature- and light-controlled room, and had free access to the food and water. All mice were euthanized by inhaled CO_2 overdose followed by cervical dislocation. Following euthanization, blood was collected for serology test. Different tissues according to the tropisms of the seven pathogens investigated in this study were collected to investigate the infectious status of mice. These tissues were

mesenteric lymph node (MLN) or spleen for mouse parvovirus, trachea for *Mycoplasma pulmonis*, oral swab from nasopharynx for *P. pneumotropica*, and feces for *Helicobacter*, mouse hepatitis virus (MHV), murine norovirus (MNV), and Theiler's murine encephalomyelitis virus (TMEV). Semen, oocytes, embryos, and ovarian tissues were collected to check contamination status in them. After collection, blood was directly sent to the serology laboratory in RADIL. Tissues and reproductive cells were stored in -80^0C freezer. Then total DNA and RNA were extracted from saved materials and PCR/RT-PCR assay were performed to detect each pathogen that will be described in this section.

Semen collection:

Ten-weeks-old Sencar mice were used for semen collection because previous studies in our lab showed that male Sencar mice at ten weeks had the highest prevalence of MPV contamination in semen. The following procedure was used for semen collection. Abdominal cavity was opened cauda epididymides were disected, removing as much fat as possible. The vasa deferentia close to the cauda epididymides were cut and washed two times in the working solution made of Dulbecco's phosphate buffered saline (DPBS, containing 1 g/L D-glucose and 36 mg/L sodium pyruate) (Gibco, Grand Island, NY) with 4 mg/ml Albumin, Bovine, 96-99% (BSA, Sigma, St. Louis, MO). Placed cauda epididymides with the vas deferens in a Petri dish with 1 ml of this same working solution, gently squeezed out the sperm in vas deferens and make 2-3 cuts on the cauda epididymides. Incubated at $37°$C for 15 minutes to allow the sperm swim out.

Finally semen was collected and saved in -80°C freezer for the future DNA/RNA extraction.

Oocyte-cumulus complex collection

Four- and six-weeks-old female Sencar mice were used for oocyte collection. In order to get as many oocytes as possible to have enough materials for the DNA and RNA extraction, female mice were superovulated. The steps for collecting oocytes were listed as follows: 7.5 IU pregnant mare serum gonodotropin (PMSG, Calbiochem, San Diego, CA) was introperitoneally injected to each female mouse. 48 hours post PMSG injection, 7.5 IU human chorionic gonodotropin (hCG, Calbiochem) was introperitoneally injected to each mouse. After approximate 15 hours, female mice were euthanized. Oviducts were collected. Oviducts were washed in DPBS with 4 mg/ml BSA two times. Tore the swollen ampulla of the oviduct with fine forceps and a needle and oocyte-cumulus complex were collected and successively transferred into Petri dish containing three 100 ul drops of DPBS with 4 mg/ml BSA for three washes. Equally separated the oocyte-cumulus complex into two tubes and stored in -80°C freezer. One tube used for the DNA extraction and the other for RNA extraction.

Embryo collection at morula stage:

Superovulate female mice as described previously in the section of oocyte-cumulus complex collection. After hCG injection, one male and one female mouse were placed in the same cage for overnight. The next morning, checked the vaginal plug. Male mice were euthanized. Two days after the plug check (60-72 hours

after mating), female mice were euthanized. The abdominal cavity is opened and oviducts with uterus above the cervix were dissected. The oviducts with uterus were washed two times in DPBS with 4 mg/ml BSA. The Hamilton flushing needle is put (Hamilton Company, NV) into the opening of the oviducts and flushed out morula-stage embryos in 100 ul DPBS with 4 mg/ml BSA. Embryos were washed three times in 100ul DPBS plus 4mg/ml BSA and collected into two tubes and tubes were saved in -80°C freezer for future DNA/RNA extraction.

Ovarian tissue collection:

Euthanize the female Sencar mouse. Open the abdominal cavity. Ovarian tissue including ovary and oviduct was collected from the Sencar mouse and saved in -80°C freezer for future DNA/RNA extraction.

DNA/RNA extraction:

Total DNA and RNA were extracted from tissues, semen, oocyte-cumulus complex and embryos by using DNA/RNA kits as described below. Total DNA was extracted from mouse semen, oocyte-cumulus complex and embryo using the QIAamp DNA Micro Kit (Qiagen, CA). Total RNA was extracted using the RNeasy Micro Kit (Qiagen, CA). DNA was extracted for detecting mouse parvovirus, *Helicobacter*, *M. pulmonis* and *P. pneumotropica*. RNA was extracted for detecting murine norovirus, mouse hepatitis virus and Theiler's murine encephalomyelitis virus (TMEV). In addition, to confirm infectious status of each mouse, various tissues were collected and DNA/RNA were also extracted. Tissue was collected based on the known tropism of

each pathogen. Total DNA was extracted from tissues and feces using MagAttract

DNA Mini M48 kit (Qiagen, CA) and total RNA was extracted from tissues using

MagAttract RNA Mini M48 kit (Qiagen, CA) following the manufacture's instructions.

The extraction process was done in the BioRobot M48 (Qiagen, CA).

PCR/RT-PCR assay for pathogen detection

PCR/RT-PCR assays were conducted using primers described in Table 1-2. All

reactions were performed in a GeneAmp PCR System 2720 thermocycler (Applied

Biosystems, Foster City, CA).

PCR reaction was used to detect mouse parvovirus, *Helicobacter*, *P.

pneumotropica and M. pulmonis*. The total reaction volume is 50 ul mixture containing

1.25 units of Taq DNA polymerase (Roche, Indianapolis, IN), 200 uM of each dNTP, 1

uM of each primer, 5 ul 10×buffer containing 500 mM Tris/HCl, 100 mM KCl, 50 mM

$(NH_4)_2SO_4$, 20 mM $MgCl_2$ (Roche, IN) and 5 ul of DNA solution. PCR parameter for

each pathogen is showed in Table 1-3.

RT-PCR was used to detect three RNA viruses: murine norovirus, mouse

hepatitis virus and Theiler's murine encephalomyelitis virus. Following RT-PCR,

nested PCR was used to increase the sensitivity for detection of each RNA virus.

RT-PCR was performed using the Onestep RT-PCR kit (Qiagen, CA). The total

reaction volume was 50 ul mixture containing 400 uM of each dNTP,   10 ul

5×RT-PCR buffer containing *Tris·Cl,   KCl*, $(NH_4)_2SO_4$, 12.5 mM $MgCl_2$ and DTT,

5×Q-solution 10 ul, 2 ul Onestep enzyme mix containing 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% (v/v) Nonidet® P-40, 0.5% (v/v) Tween® 20, 50% glycerol (v/v), stabilizer, 0.6 uM of each primer and 2 ul of RNA sample. RT-PCR parameter for each pathogen is shown in Table 1-4. The condition of nested PCR was same as described for PCR for DNA pathogens except 1.5 ul product from the RT-PCR was used and total volume is 46.5 ul. Parameter for each pathogen is showed in Table 1-5. After PCR/RT-PCR, amplicons underwent 3% agarose gel electrophoresis, ethidium bromide staining, and visualization under ultraviolet light.

Table 1-2 Primers used to amplify seven pathogens investigated in this study

Pathogen	Primer sequence (5'→3')		
MPV[1]	Forward: GCA GCA ATG ATG TAA CTG AAG CT		
	Reverse: CCA TCT GCC TGA ATC ATA GCT AA		
Helicobacter[2]	Forward: CTA TGA CGG GTA TCC GGC		
	Reverse: ATT CCA CCT ACC TCT CCC A		
M. pulmonis[3]	Forward: AGC GTT TGC TTC ACT TTG AA		
	Reverse: GGG CAT TTC CTC CCT AAG CT		
P. pneumotropica[4]	PpJL	Forward: TAA GGA CAA AAG GGG GCG TA	
		Reverse: GGG TAT TAA CCT TAT CAC C	
	PpHL	Forward: ACC GCC TAA AGT CTT TGG AC	
		Reverse: AAC GTC AAT CAG CTT GGC TA	
MNV	Primary primer set[5]	Forward: CAG ATC ACA TGC TTC CCA C	
		Reverse: AGA CCA CAA AAG ACT CAT CAC	
	Nested primer set	Forward: ACC AGG CGC ATA GAT TCC T	

		Reverse: CAC ATG CTT CCC ACA TGT C
MHV	Primary primer set[6]	Forward: GAG ACT ATC ATG AAA GTG TTG AAT G
		Reverse: TAC ACA TTA GAG TCA TCT TCT AA
	Nested primer set	Forward: GAT GAA GTA GAT AAT GTA AGC GTT G
		Reverse: TAC ACA TTA GAG TCA TCT TCT AA
TMEV	Primary primer set	Forward: CTA ATC AGA GGA ACG TCA GC
		Reverse: CTT AGA TCT CCA ACC ACG TC
	Nested primer set	Forward: GCC CAG GCT AAG AGA AGT A
		Reverse: CAG GGG CCG AAT TAG AAT G

1. *Besselsen et al., 1995*

2. *Riley et al., 1996 (Helicobacter generic)*

3. *van Kuppeveld et al., 1992 (M. pulmonis)*

4. *Two primer sets for two biotypes of P. pneumotropica: Jawetz and Heyl. PpJL primer set is for Jawetz and PpHL is for Heyl.*

5. *Hsu et al., 2005*

6. *Matthaei et al., 1998*

Table 1-3 PCR parameters for MPV, *Helicobacter*, *P. pneumotropica* and *M. pulmonis*

		MPV	*Helico.*[1]	*P. p*[2]	*M. p*[3]
Initial denaturation	temperature	95°C	95°C	95°C	95°C
	time	4 min.	4 min.	15 min.	4 min.
Denaturation	temperate	94°C	94°C	94°C	94°C
	time	10 sec.	30 sec.	20 sec.	10 sec.
Annealing	temperature	57°C	62°C	60°C	63°C
	time	10 sec.	30 sec.	20 sec.	10 sec.
Extension	temperature	72°C	72°C	72°C	72°C
	time	30 sec.	30 sec.	30sec.	30 sec.
Final extension	temperature	NA	72	NA	NA
	time	NA	7 min.	NA	NA
Hold	temperature	4°C	4°C	4°C	4°C
	time	∞	∞	∞	∞
Cycle number[4]		45	45	35	40

1. *Helicobacter*

2. *Pasteurella pneumotropica*

3. *Mycoplasma pulmonis*

4. Cycle number refers to times needed to repeat from step of denaturation to extension

Table 1-4 Primary RT-PCR parameters for murine norivirus (MNV), mouse hepatitis virus (MHV), Theiler's murine encephalomyelitis virus (TMEV)

		MNV	MHV	TMEV
Reverse transcription	temperature	50°C	50°C	50°C
	time	30min.	30min.	30min.
Initial denaturation	temperature	95°C	95°C	95°C
	time	15min.	15min.	15min.
Denaturation	temperature	94°C	94°C	94°C
	time	30sec.	30sec.	30sec.
Annealing	temperature	57°C	56.5°C	53°C
	time	1min.	1min.	1min.
Extension	temperature	72°C	72°C	72°C
	time	2min.	2min.	2min.
Final extension	temperature	72°C	72°C	72°C
	time	10min.	10min.	10min.
Hold	temperature	4°C	4°C	4°C
	time	∞	∞	∞
Cycle number[1]		40	40	40

1. Cycle number refers to times needed to repeat from step of denaturation to extension.

Table 1-5 Nested PCR parameters for murine norivirus (MNV), mouse hepatitis virus (MHV), Theiler's murine encephalomyelitis virus (TMEV)

		MNV	MHV	TMEV
Initial denaturation	Temperature	95°C	95°C	95°C
	Time	4min.	4min.	4min.
Denaturation	Temperature	94°C	94°C	94°C
	Time	10sec.	10sec.	10sec.
Annealing	Temperature	58°C	56°C	58°C
	Time	10sec.	10sec.	10sec.
Extension	Temperature	72°C	72°C	72°C
	Time	30sec.	30sec.	30sec.
Hold	Temperature	4°C	4°C	4°C
	Time	∞	∞	∞
Cycle number[1]		40	40	40

1. Cycle number refers to times needed to repeat from step of denaturation to extension.

2. Results

2.1 Pathogens in mouse semen

In the initial experiment, fifteen Sencar male mice at 10-weeks of age were evaluated for seven microbial pathogens including mouse parvovirus, *Helicobacter*, *M. pulmonis*, *P. pneumotropica,* murine norovirus, mouse hepatitis virus and Theiler's murine encephalomyelitis virus. Previous data indicated that 10-weeks-old male Sencar mice had the highest prevalence of MPV contamination in semen, so MPV was one of the pathogens of greatest interest. Five pathogens except *M. pulmonis* and *P. pneumotropica* were detected in the semen of these fifteen mice at different infectious prevalence by PCR test. For this reason, in our following studies we focused on the five pathogens found in the Sencar mouse semen.

In the second experiment, forty additional mice were evaluated.   In total, fifty five male Sencar mice at ten weeks of age were tested for the presence of microbial pathogens in semen. The results are summarized in Table 1-6. Of the fifty five animals evaluated, the 94.5% of mice were positive for MPV, 100% of mice were positive for *Helicobacter*, 98.2% of mice were positive for MNV, 94.5% of mice were positive for TMEV indicating these four pathogens were common in the Sencar mouse. In contrast, only fifteen Sencar mice were infected by mouse hepatitis virus.

As shown in table 1-6, mouse parvovirus, *Helicobacte*r, murine norovirus, mouse hepatitis virus and Theiler's murine encephalomyelitis virus were all detected in the Sencar mouse semen: 65.45% of mouse semen was positive for mouse parvovirus,

14.5% of mouse semen was positive for *Helicobacter*, 14.5% of mouse semen was positive for Murine norovirus, 3.6% of mouse semen was positive for Mouse hepatitis virus, and 20% of mouse semen was positive for Theiler's Murine Encephalomyelitis virus. The results suggested that mouse parvovirus had the highest infectious prevalence in the semen of the Sencar mice. *Helicobacter*, murine norovirus, and Theiler's murine encephalomyelitis virus had similar infectious prevalence while mouse hepatitis virus had the lowest infectious prevalence. Additional studies describing mouse parvovirus contamination in mouse semen will be shown following this section.

Table 1-6 Summary of microbial pathogen contamination in Sencar mouse semen

Pathogens	No. of infected animals/No. tested	No. of mice with contaminated semen/No. tested	% of semen samples contaminated
MPV	52/55	36/55	65.45
Helicobacter	55/55	8/55	14.5
MNV	54/55	8/55	14.5
MHV	14/55	2/55	3.6
TMEV	52/55	11/55	20
P. pneumotropica	13/15	0/15	0
M. pulmonis.	5/15	0/15	0

2.2 Pathogens in mouse oocyte-cumulus complexes

Two groups of female Sencar mice at different ages were euthanized for studying microbial pathogens in mouse oocyte-cumulus complex. One group was 4-weeks-old and the other group was 6-weeks-old. The results were summarized in Table 1-7 and Table 1-8.

As shown in table 1-7, among twenty-one 4-week-old female Sencar mice, mouse parvovirus was found in only two mice based on PCR and serology testes as compared to a much higher percentage of infections by the other 6 pathogens: *Helicobacter*, *M. pulmonis, P. pneumotropica,* murine norovirus, mouse hepatitis virus, murine norovirus and Theiler's murine encephalomyelitis virus. The possible reason for the low infection prevalence of mouse parvovirus was that the younger mice still possess maternal antibody which prevents infection by same microbial pathogens. In the oocyte-cumulus complexes collected from 4-weeks-old female Sencar mice, 4.8% of the oocyte-cumulus complex was mouse parvovirus positive, 10% was *Helicobacter* positive, and 19% was positive for murine norovirus. None of these oocyte-cumulus complexes was positive for mouse hepatitis virus, Theiler's murine encephalomyelitis virus, *P. pneumotropica* and *M. pulmonis.*

In table 1-8, of the fifteen 6-weeks-old female Sencar mice examined, all were infected by mouse parvovirus, compared to very low percentage of infection in 4-weeks-old mice. The infection prevalence was also high for other viruses except for mouse hepatic virus. This showed that the 6-weeks-old mouse was a better model for the study of microbial pathogen study in mouse gametes and embryos. This

phenomenon may be caused by the disappearance of maternal antibody, Sencar mice do not have protection from pathogens. In oocyte-cumulus complex collected from the 6-weeks-old female Sencar mice, 73.3% of mouse oocytes were positive for mouse parvovirus, 20% of oocytes were positive for *Helicobacter*, 6.7% of oocytes were mouse hepatitis virus positive, and 13.3% of oocytes were murine norovirus positive. Same as the samples collected from 4-weeks old female Sencar mice, none of oocytes were positive for *M. pulmonis*, *P. pneumotropica* and Theiler's murine encephalomyelitis virus. These data indicated that mouse oocyte-cumulus complexes collected from Sencar mice were contaminated by different pathogens which suggested that cryopreserved oocyte-cumulus complexes may carry pathogens. Comparing two-age groups of female Sencar mice investigated in this study, 6-weeks-old female Sencar mouse was a better animal model to study microbial pathogens contamination in mouse oocyte-cumulus complexes because this age gave higher infectious prevalence.

Table 1-7 Summary of microbial pathogen contamination in oocyte-cumulus complex of 4-weeks-old Sencar mouse

Pathogens	No. of infected animals/No. tested	No. of mice with contaminated oocytes/No. tested	% of oocyte contaminated
MPV	2/21	1/21	4.8
Helicobacter.	21/21	2/21	9.5
MNV	21/21	4/21	19
MHV	16/21	0/21	0
TMEV	21/21	0/21	0
P. pneumotropica	20/21	0/21	0
M. pulmonis	11/21	0/21	0

Table 1-8 Summary of microbial Pathogens in oocyte-cumulus complex of

6-weeks-old Sencar mouse

Pathogens	No. of infected animals/No. of tested	No. of mice with contaminated oocytes/No. of tested	Percentage of oocyte contaminated
MPV	15/15	11/15	73.3
Helicobacter	15/15	3/15	20
MNV	15/15	2/15	13.3
MHV	2/15	1/15	6.7
TMEV	15/15	0/15	0
P. pneumotropica	13/15	0/15	0
M. pulmonis	6/15	0/15	0

2.3 Pathogens in mouse embryo

2.3.1 Microbial pathogens in mouse embryo when both female and male mice were the Sencar mice

In initial experiments naturally infected male and female Sencar mice were bred, and results showed that mouse parvovirus, mouse hepatitis virus and murine norovirus were present in the embryos (Table 1-9). Among twenty-one pregnant females which produced embryos, mouse parvovirus, mouse hepatitis virus and murine norovirus were all detected in the mouse embryos: 47.6% of mice had contamination of mouse parvovirus in embryos, 28.6% of mice had contamination of mouse hepatitis virus in embryos and 19% of mice had contamination of murine norovirus in embryos. These findings indicated that the infected mice produced contaminated embryos, thus contaminated embryos may have the risk of transmitting microbial pathogens and potentially cause outbreaks of disease. Contamination in mouse embryos may come from the male mouse (semen donor) and/or the female mouse (oocyte donor). It was of interest to investigate which one played a major role in the contamination so that effective measures can be taken to avoid contamination in both *in-vivo* and *in-vitro* generated embryos. Under the circumstances that both female and male mice were contaminated, one can not determine which party played a more important role. Therefore, in the next step, two additional groups of mice were investigated. In the first group, the male mouse was the Sencar infected mouse and the female was the uninfected ICR mouse. In the second group the male mouse was the uninfected ICR mouse and the female was the Sencar infected mouse.

Table 1-9 Summary of microbial pathogen contamination in mouse morula-stage

embryos produced by Sencar male and female mice

Pathogens	No. of embryo contaminated/No. tested	Percentage of embryo contaminated
MPV	10/21	47.6
Helicobacter	0/21	0
MHV	6/21	28.6
MNV	4/21	19
TMEV	0/21	0

2.3.2 Microbial pathogens in mouse embryos when the male mouse was the Sencar mouse and the female was the ICR mouse

Twenty naturally infected male Sencar mice and twenty female uninfected ICR mice were bred. Thirteen female mice became pregnant and produced embryos. Microbial pathogens were investigated in the embryos collected from these thirteen female mice. The results are summarized in Table 1-10. It was noted that only *Helicobacter* was detected in embryos with a contamination prevalence of 25%. The other four pathogens were not detected in embryos collected from these thirteen mice.

2.3.3 Microbial pathogens in mouse embryos when the male was the ICR mouse and the female mouse was the Sencar mouse

Twenty uninfected male ICR mice and twenty naturally infected female Sencar mice were bred, seventeen female mice became pregnant and produce embryos. Microbial pathogens were investigated in the embryos collected from seventeen female mice. The results were summarized in Table 1-11. Four microbial pathogens, mouse parvovirus, *Helicobacter*, murine norovirus, and mouse hepatitis virus were detected in the embryos collected from seventeen female Sencar mice (Table 1-11). The contamination prevalence of mouse parvovirus in embryos was 35.3%. The contamination prevalence of *Helicobacter* in mouse embryos was 17.6%. The contamination prevalence of murine norovirus in mouse embryos was 11.8%. The contamination prevalence of mouse hepatitis virus in mouse embryos was 5.9%.

These data from two-group mice in 2.3.2 and 2.3.3 suggest that female mice had greater potential to transmit microbial pathogens to embryo than male mice.

Table 1-10 Summary of microbial pathogen contamination in mouse morula-stage embryos produced by Sencar male mice and uninfected ICR female mice

Pathogens	No. of embryo contaminated/No. of tested	Percentage of embryo contaminated
MPV	0/12	0
Helicobacter	3/12	25
MNV	0/12	0
MHV	0/12	0
TMEV	0/12	0

Table 1-11 Summary of microbial pathogen contamination in morula-stage embryos produced by uninfected ICR male mice and Sencar female mice

Pathogens	No. of embryo contaminated/No. of tested	Percentage of embryo contaminated
MPV	6/17	35.3
Helicobacter	3/17	17.6
MNV	2/17	11.8
MHV	1/17	5.9
TMEV	0/17	0

2.4 Microbial pathogens in mouse ovarian tissue

Seventeen Sencar female mice from the mice used for collecting embryos were used for investigating mouse parvovirus, *Helicobacter,* murine norovirus, mouse hepatitis virus and Theiler's murine encephalomyelitis virus in the ovarian tissue. Results were shown in table 1-12. 94.12% of mice had MPV contamination in ovarian tissue. 58.8% of mice had *Helicobacter* contamination in ovarian tissue. 5.9% of mice had TME contamination in ovarian tissue. These data suggest that oocytes and embyos may be contaminated when they are within ovary and as they travel through oviduct.

Table 1-12 Summary of microbial pathogen contamination in mouse ovarian

tissue collected from Sencar mice

Pathogens	No. of infected animals/No. tested	No. of mice with contaminated ovarian tissue/No. tested	% of ovarian tissue contaminated
MPV	17/17	16/17	94.12
Helicobacter	17/17	10/17	58.8
MNV	16/17	0/17	0
MHV	5/17	0/17	0
TMEV	15/17	1/17	5.9

3. Discussions and conclusions

In this study, we investigated which of the commonly found murine pathogens contaminated mouse gametes and embryo. Microbial pathogens were found in mouse gametes and embryos including: mouse parvovirus, murine norovirus, mouse hepatitis virus, Theiler's murine encephalomyelitis virus. These findings suggest that cryopreserved gametes and embryos have potential risk of disease transmission. Measures need to be taken to reduce or/and eliminate contamination.

Microbial pathogens are found associated with gametes and embryos in various species such as human immunodeficiency virus (Baccetti *et al*., 1994; Quayle *et al*.,1997; Quayle *et al*., 1998; Pudney *et al*., 1999), hepatitis B virus (Hadchouel et al., 1985; Huang *et al*., 2003), bovine herpesvirus-1 (BHV-1) and bovine viral diarrhea virus (BVDV) (Baker, 1995; Brock *et al*., 1998; Rossi *et al*., 1980), porcine reproductive and respiratory syndrome virus (Sur, 1997) and porcine parvovirus (Johnson *et al*., 1976). Genome banking will have limited value if cryopreserved gametes and embryos are contaminated with microbial pathogens. Not only will microbial pathogens adversely affect research using the regenerated line, they can also spread to other susceptible animals within the facility causing outbreaks of disease that may compromise many research investigations.

Mouse parvovirus has been found in mouse sperm, oocytes, embryos and ovarian tissue (Agca *et al.,* 2007). Another study shows that the presence of mouse hepatitis virus in fertilization, culture, and embryo transfer media causes

seroconversion in mother recipient and offspring. Moreover, the cleavage rate is significantly lower if the virus is present in the fertilization medium (Peters *et al.*, 2006). Therefore, based on previous findings, the possibility of presence and transmission of other common murine pathogens such as *Helicobacter* and murine norovirus cannot be excluded. In this study, we investigated seven microbial pathogens in mouse semen, oocyte-cumulus complex and embryos collected from Sencar mouse naturally infected with the following pathogens: mouse parvovirus, *Helicobacter*, murine norovirus, mouse hepatitis virus, Theiler's murine encephalomyelitis virus, *Mycoplasma pulmonis* and *Pasteurella pneumotropica*.

Except for *M. pulmonis* and *P. pneumotropica*, the other five microbial pathogens were all detected in mouse semen with mouse parvovirus having the highest prevalence of infection at 65.45%. The fact that multiple microbial pathogens were found in mouse semen indicated that mouse semen serves as a potential source for transmitting various diseases. Contamination of semen has been reported to cause genital infections of the female recipient, which can decrease fertilization rates, alter embryo development, and result in infected offspring (Guillet-Rosso *et al*., 1987; Hewitt *et al.,* 1985). However, it is not clear where these microbial pathogens are located. They may associate with spermatozoa through either attachment to the sperm surface or existing inside the cytoplasm of the spermatozoa (Elazhary *et al.*, 1980; Van Engelenburg *et al*., 1993; Baccetti *et al.*, 1994). They may exist in seminal fluid, or other non-sperm cell components, such as epithelial cells, lymphocytes, leukocytes and macrophages (van Oirschot 1995; Nash *et al.*, 1995). The standard preparation of

semen for artificial insemination (AI) involves the dilution of ejaculates to achieve an appropriate concentration of sperm in each insemination dose. This procedure decreases the concentration of contaminants, and to some degree it may minimize the risk of disease transmission. Washing procedures such as centrifugation, swim-up or Percoll gradients used to separate the highly motile sperm fraction from the seminal plasma also reduce microbial contaminants associated with sperm (Nicholson *et al.*, 2000; Hanabusa *et al.*, 2000; loskutoff *et al.*, 2005; Wong *et al.*, 1986; Cottell *et al.*, 1997) The complete elimination of viruses penetrating into the sperm may be difficult or impossible to achieve (Bielanski, 2007).

Two factors were considered when selecting the female mouse at appropriate age for detection of microbial pathogen contamination in oocyte-cumulus complexes. One factor is to choose the mouse at its optimal age for PMSG and hCG response because these two hormones were used to superovulate female mice. The other factor to consider is the age at which mouse oocytes may become contaminated with microbial pathogens. Different mouse strains respond to PMSG and hCG in a different way at the different age. But typically 3- to 6-weeks-old mice exhibit optimal hormone responses (Hogan *et al.,* 1994). Between the two different age group mice investigated, 4-weeks-old female Sencar mice were contaminated by mouse parvovirus at lower prevalence compared to 6-weeks-old mice. This was likely due to the presence of maternal antibody which prevents infection. For 6-weeks-old female Sencar mouse (n=15), 100% of mice were documented to be infected by mouse parvovirus. Therefore, the 6-weeks-old female Sencar mouse proved to be a better model for the study of

microbial contamination of oocytes. Of the fifteen 6-weeks-old female mice examined, 73.3% were positive for mouse parvovirus in oocyte-cumulus complex, much higher than those for *Helicobacter* (20%), murine norovirus (13.3%) and mouse hepatitis virus (6.7%).

The detection of these pathogens in mouse oocyte-cumulus complex collected from naturally infected Sencar mouse indicated the possibility of infection transmission through the oocytes collected from infected donor animals. Each ovulated oocyte is surrounded by its zona pellucida and a mass of follicle cells (cumulus cells). Studies of contamination in oocytes in other species showed that contamination may occur at several stages. Prior to ovulation, oocytes may become contaminated by contact with an infectious agent present in either the ovarian cumulus cells or the follicle fluid, probably during viremia at the acute stage of disease (Fray *et al.*, 2000; Brownlie *et al.*, 1997; Bielanski and Dubuc, 1994; Bielanski *et al.*, 1998). Following ovulation, oocytes may be contaminated by exposure to pathogens that exist in the oviduct or uterus (Booth *et al.*, 1995).

Both *P. pneumotropica* and *M. pulmonis* were not detected in mouse semen and oocyte-cumulus complex although the majority (90%) of the investigated mice was infected by *P. pneumotropica* and 43% of the investigated mice were infected by *M. pulmonis.* The possible reason is they are not sexually transmitted pathogens. Another possible reason is these two pathogens do contaminate male and female reproductive system, but it is intermittent so they do not show up in the time period the semen and oocyte-cumulus were collected in this study. While 20% of mouse semen was positive

for TMEV, no mouse oocyte-cumulus complex collected from the investigated mice in this study was positive for TMEV. This suggests that semen is more likely to be contaminated with TMEV than oocyte–cumulus complex.

Microbial pathogens were detected in mouse embryos at morula-stage indicated that embryos could also be the potential source to transmit microbial pathogens when contaminated embryos were collected, stored and rederived for specific research. Among the five pathogens investigated in this study, mouse parvovirus, mouse hepatitis virus and murine norovirus were found in embryos produced by Sencar male and female mice. *Helicobacter* is the only pathogen found in embryos produced by infected Sencar male mice and uninfected ICR female mice. Mouse parvovirus, *Helicobacter*, murine norovirus and mouse hepatitis virus were found in the embryos produced by uninfected ICR male mice and infected Sencar female mice. These data suggest that the female has greater potential to transmit pathogens to the embryos than male mice. Theiler's murine Encephalomyelitis virus was not detected in mouse embryos no matter what was the male and female mice infectious status.

In this study, we focused on the embryos produced *in vivo*. As assisted reproductive technologies are increasingly used to regenerate transgenic animal lines, a large frequency of the embryos is generated *in vitro*. The risk of microbial pathogen transmission is higher in *in-vitro* derived embryos than in *in-vivo* derived embryos. This has been documented for several livestock pathogens including foot-and-mouth disease (Marquant *et al.,* 1998) and Bovine herpes virus (Bielanski *et al.,* 1997). The increasing risk in pathogen transmission in *in-vitro* derived embryos may be due to the

fact that the normal female genital tract defenses are averted in IVF (Kim *et al.*, 1998) or because *in vitro*-derived embryos are morphologically and physiologically different from *in vivo*-derived embryos (Massip *et al.*, 1995; Wright and Ellington, 1995). The contamination status in mouse embryos generated *in vitro* needs to be considered and evaluated.

Like oocytes, embryos may become contaminated by microbial pathogens at the oocyte stage prior to ovulation. Or embryos may be contaminated following ovulation by coming in contact with microbial pathogens during transit through the oviduct or after arrival at the uterus. Embryos may be contaminated by spermatozoa (Baccetti *et al.*, 1994). Oocytes and embryos are surrounded by the zona pellucida which protects them from physical injury and infection. In general, the ZP of mammalian species is composed of 3 glycoproteins, which are assembled in a complex three-dimensional structure. Changes in the structure of the glycoproteins occur during fertilization and also during the passage of the embryo through the oviduct (Tallec et al., 2001). The specific structural and chemical nature of the ZP is a major factor with regard to its interaction with pathogenic microorganisms and its role in disease transmission (Stringfellow et al., 1999). Washing procedures for *in vivo*-derived embryos in the presence of antibiotics and trypsin are adopted and endorsed by the International Embryo Transfer Society (IETS) and the Office International des Epizooties (OIE) as a standard sanitary requirement for health certification of livestock embryos (Stringfellow and Seidel, 1998). This procedure has been showed to be effective in rendering bovine, porcine and ovine *in vivo* derived ZP-intact embryos free

of a number of viruses and bacteria (Wrathall *et al.*, 2002). However some microbial pathogens that adhere firmly to the ZP may not entirely be removed using this described methodology ((Stringfellow and Seidel, 1998).

CHAPTER 2 EVALUATE MOUSE PARVOVIRUS TRANSMISSION TO EMBRYOS AND EFFECT ON FERTILIZATION AND EMBRYONIC DEVELOPMENT DURING *IN VITRO* FERTILIZATION WITH INFECTED MALE MICE

I. General information

1. General information about mouse parvovirus

Mouse parvovirus (MPV) is one of the most prevalent infectious agents in laboratory mice (Jacoby and Lindsey, 1998; Livingston and Riley, 2003), as also found in our studies. Parvovirus is a small (15-28 nm) nonenveloped icosahedra that encapsidates a single stranded DNA genome approximately 5 kb in length. Parvovirus infection in swine can cause stillborn and mummified piglets and a decrease in the litter size (van Leengoed *et al.*, 1983). Porcine parvovirus enters pores in the zona pellucida (ZP) of the embryo and is seen enmeshed in cellular debris on the outer surface of the ZP (Bolin *et al.,* 1983). Clinical diseases and histological lesions have not been observed in mice naturally or experimentally infected with MPV (Jacoby *et al.*, 1995; Smith *et al.*, 1993). However, this virus can have deleterious effects on research due to *in vitro* and *in vivo* immunomodulatory effects, tumor suppression and contamination of cell culture and tissues originating from mice (McKisic *et al.*, 1993; 1996, 1998**)**. Mouse parvovirus is found in mouse sperm, oocyte, embryo and ovarian tissues (Agca *et al.,* 2007). From our studies in Chapter 1, mouse parvovirus is the most prevalent infectious agent among the five pathogens found in mouse gametes

and embryos, therefore, in this part of the investigation, we focused on MPV to study whether it transmits to embryos produced *in vitro* and the effect on fertilization and embryonic development during *in vitro* fertilization with infection of male mice.

2. General information about in vitro fertilization

In vitro fertilization (IVF) is a method in which oocytes are fertilized by sperm outside of the female uterus. Fertilization encompasses a series of events that result in the fusion of the male and female gametes: 1) contact with and penetration of the cellular investments of an oocyte by a spermatozoa; 2) penetration of the ZP; 3) fusion of the spermatozoa and oocyte external membranes, and 4) pronuclei fusion and alignment of their respective chromosomes on the first cleavage spindle. Ultrasound studies have documented each of these physiological events (Austin, 1968; Bedford, 1970; Zamboni, 1971; Gould, 1975; Gwatkin, 1977). With the development of sperm cryopreservation technology, *in vitro* fertilization becomes one of the most important assisted technologies to rederive animals from cryopreserved sperm. IVF has been frequently used for treatment of sterility in humans and improve the quality and increase production in cattle. In laboratory animals, IVF has also been frequently used to improve the reproductive ability of mutant or aged mice (Thornton *et al.,* 1999; Marschall *et al*., 1999; Nakagata, 1994; Wakayama *et al*., 1995; Sztein *et a.l*, 2000; Nagy *et al*., 2003; Marschall *et al*., 2003). A lot of researches focusing on effects of media and mouse strains on the results of IVF were reported (Loutradis *et al.,* 2000; Kaneda *et al.,* 2007). This study investigated effects of MPV on IVF.

3. General information about microbial pathogen contamination in the procedure of *in vitro* fertilization

In the first part of this study, microbial pathogens were found associated with *in vivo* derived mouse embryos. This section focused on *in vitro* derived embryos. The risk of microbial pathogen transmission is higher in *in-vitro* derived embryos than in *in-vivo* derived embryos. This has been documented for several livestock pathogens including foot-and-mouth disease (Marquant *et al.,* 1998) and Bovine herpes virus (Bielanski *et al.,* 1997). The increased risk in pathogen transmission in *in-vitro* derived embryos may be due to the fact that the normal female genital tract defenses are averted in IVF (Kim *et al*., 1998) or because *in vitro*-derived embryos are morphologically and physiologically different from *in vivo*-derived embryos (Massip *et al*., 1995; Wright and Ellington, 1996). Evidence indicates that there is low potential for transmission of pathogens with *in-vivo* derived embryos of cattle when appropriate precautions are taken (Baker, 1995; Stringfellow and Givens, 2000). In contrast, washing and trypsin treatment are ineffective for removing some viruses from bovine embryos produced *in vitro* such as bovine herpesvirus-1 and bovine diarrhea virus (Bielanski and Dubuc, 1993; Trachte *et al*., 1998), and bluetongue virus (Langston *et al.,* 1999).

Microbial pathogens may potentially originate from the donor male (semen) or the donor female (oocytes and/or embryos). Contamination can be present in various environments, such as serum or media used for the handling of oocytes and embryos. Pathogens may also be added during the whole process of embryo production, from collection to transfer to storage (Tallec *et al*., 2001a). Many pathogens have been

reported to occur in the semen of cattle and other livestock (Afshar and Eaglesome, 1990; Eaglesome and Garcia, 1997; Philpott, 1993), so among these multiple factors that may cause contaminated embryos, semen is one of the important sources. Bacteria and viruses have been reported to transmit to embryos derived through IVF. Bacteria were isolated from the culture medium of zygote derived from IVF using bull semen contaminated with various species of common bacteria such as *Staphylococcus sciuri, Enterobacter cloacae, Pantoea agglomerans* (Kim *et al.*, 1998). Use of semen infected with bovine viral diarrhoea virus (BVDV), bluetongue virus (BTV), enzootic bovine leukosis virus (EBLV), bovine herpesvirus-1 (BoHV-1), with the probable exception of EBLV, will often lead to contaminated embryos, and virus removal from these embryos is difficult even when the internationally approved embryo processing protocols are used (Trachte *et al.*, 1998; Langston *et al.*, 1999; Bielanski and Dubuc, 1994; Van Der Maaten and Miller, 1978; Bielanski *et al.*, 2000; Wrathall, 2006).

Not only contaminated semen transmits microbial pathogens to embryos but also affects *in vitro* fertilization. Bielanski and Stewart (1996) reported that embryonic development was suppressed by ubiquitous bacteria and fungi such as *Corynebacterium, Streptococcus, Staphylococcus, Micrococcus, Pseudomonas* and by yeast present in maturation and co-culture media of the IVF system. Further, Stringfellow *et al.* (1997) reported that contamination of matured oocytes with *Stenotrophomonas maltophilia* by semen during IVF resulted in degeneration of cleaved embryos. Vanroose *et al.* (1999) found that rates of cleavage and blastocyst

formation were significantly reduced by exposure to the virus during the IVF stage of *in vitro* embryo production. Guerin *et al*. (1992) found that fertilization and embryo cleavage rates were significantly reduced when semen from a BVDV persistently infected bull was used to inseminate oocytes, and development to the blastocyst stage was only 2.1% compared to 19.6% with uninfected, control bull semen.

II. Evaluation of Mouse parvovirus transmission to embryos and effect on fertilization and embryonic development during *in vitro* fertilization with infected male mice

1. Material and method:

Virus:

Two vials of Mouse Parvovirus-1 (MPV-1) were generously provided by Steve Jennings (Charles River Laboratory, Wilmington). To make this seed, feces and mesenteric lymph nodes were collected from naturally infected animals and homogenized in Hanks' medium. Then this homogenate was inoculated into four weeks old BALB/c mice, 50 ul orally, and 100 ul introperitoneally per mouse. At day four post inoculation, inoculated mice were euthanized and their spleens and mesenteric lymph nodes (MLN) were removed. The spleen and lymph nodes were homogenized in Hanks' medium (10% weight/volume) and divided into the aliquots which was the virus provided by Steve Jennings. In order to get more viruses from the two vials of MPV-1 field isolate, four weeks old BALB/C mice from NIH were used to inoculate this virus following Steve Jennings' protocol.

Mice:

ICR mouse colony from Harlan (Indianapolis, IN) was used in this study. This colony was routinely subjected to health monitoring by the Research Animal Diagnosis Laboratory (RADIL) at the University of Missouri-Columbia. Results

confirmed that this colony was free of common murine microbial pathogens including mouse parvovirus investigated in this study. Male ICR mice were inoculated with mouse parvovirus-1 50 ul orally and 100 ul introperitoneally per mouse and then were housed in the animal facility for infected mice. Control mice were male ICR mice without inoculation and housed in the animal facility for clean animals. Oocyte donors were 4-weeks-old uninfected ICR mice.

Semen collection:

To collect semen, cauda epididymis and vas deferens were dissected using small spring scissors and curved tweezers and quickly placed into a 35 mm Petri dish with FHM (mouse embryo handling medium) containing 4 mg/ml Albumin, Bovine, 96-99% (BSA, Sigma, MO). The blood vessels attached to the vas deferens and any fat associated with the cauda epididymis were removed using sharp point tweezers and microscissors. Vas deferens were cut from the cauda and transferred to a 100 ul drop of HTF (sperm capacitation and mouse in vitro fertilization medium) contains 4 mg/ml BSA that had been equilibrated overnight in the incubator. Semen was gently squeezed from the vas deferens into the drop using sharp point tweezers and microscissors, and tissue was discarded. Cauda epididymis was placed into the same 100 ul drop of HTF medium and one slice was carefully cut through it using microscissors and sharp point tweezers. This process should be done very carefully not to introduce blood or fat into HTF medium. This procedure was repeated to collect sperm from the other side of cauda epididymis and vas deferens. Petri dish with HTF and mouse sperm was paled into the incubator for 15-20 minutes to let the

sperm swim out of the medium. Then cauda epididymis tissues were removed and Petri dish with sperm was placed back into incubator for approximately 1 hour to let the sperm capacitate.

Oocyte-cumulus complex collection:

ICR female mouse at 4-weeks-old was intraperitoneally injected with 5 IU of Pregnant mare serum gonodotropin (PMSG). 5 IU of human chorionic gonodotropin (hCG) was injected to these same female mice after forty eight hours. Eleven hours post hCG injection, sperm collection was started (see sperm collection section).

Before collecting sperm, an FHM working solution for cumulus cell mass, cauda epididymis and vas deferens washing was prepared. FHM was placed in 37°C water bath for 30 minutes prior to sperm collection. Female ICR mice at 4-weeks-old were sacrificed thirteen hours post hCG injection and ½ hour before the end of sperm capacitation. Reproductive tracts were dissected, removing only the oviducts into one labeled 35 mm dish of FHM. The clutch of oocytes was carefully expelled using 27g needle and sharp point tweezers and washed through one dish of FHM using a Hamilton pipette and gas-tight plunger (Hamilton Company, Nevada). Oocye-cumulus complexes were washed through two 100 ul fertilization drops of HTF medium, and placed into the third drop.

Fertilization

First the percentage of motile mouse sperm was determined with the aid of a computer assisted semen analyzer (Hamilton-Thorne analysis system, model HTM-IVOS, version 10). The motile sperm concentration was used to determine the volume of sperm to add to 100 ul fertilization drop of HTF medium with collected oocyte-cumulus complex mentioned in "Oocyte-cumulus complex collection" section. This calculation gave microliters of sperm needed to transfer to achieve a 1 million motile sperm per milliliter concentration. The calculated volume of capacitated motile sperm solution was added into 100 ul fertilization drop of HTF medium with oocyte-cumulus complex and the Petri dishes were returned to the incubator right away. If the volume added exceeded 10ul, be sure to remove an equal volume of the fertilization drop to keep the volume the same. Fertilization medium with sperm and oocyte-cumulus was left in incubator for six hours.

Fertilization assessment and embryo culture

Six hours after adding semen to fertilization medium, presumptive zygotes were removed from fertilization medium using a glass transfer pipette and washed through two drops of KSOM (culture medium) containing 1 mg/ml BSA to remove as much attached sperm as possible. Pronuclei and polar bodies were checked at this time using an inverted microscope (×300 magnification). All presumptive zygotes were transferred into one KSOM drop. Make sure not to transfer degenerating oocytes into culture drop. Fresh KSOM drops was made and placed into incubator overnight

for equilibration. Twenty four hours after IVF, two-cell embryos were checked and transferred to a fresh drop of KSOM which was equilibrated overnight. Development of blastocysts were checked four days after IVF.

DNA extraction

Total DNA was extracted from spleen, mesenteric lymph node (MLN) and feces by using DNeasy Tissue Kit (Qiagen, CA). Total DNA was also extracted from blastocysts, abnormal and degenerated embryos by using QIAamp DNA Micro Kit (Qiagen, CA).

PCR

PCR was done as mentioned in the first part of this study to investigate MPV-1 infection in mouse and contamination in blastocysts and degenerated embryos by using the same primers and same parameter. Amplicons underwent 3% agarose gel electrophoresis, ethidium bromide staining, and visualization under ultraviolet light.

Serologic test

Serology test was done by Research Animal Diagnosis laboratory (RADIL) in University of Missouri-Columbia by multiple fluorescent immunoassay technology.

Statistical method

Student t-test was used for evaluating difference in fertilization, cleavage rate and embryonic development. Differences at a probability of less than 0.05 were considered to be significant.

2. Result

2.1. Mouse parvovirus-1 infection and contamination in semen in the pre-IVF mice

A total of fourteen 4-weeks-old male ICR mice were ordered from Harlan. One mouse was euthanized before mouse parvovirus-1 inoculation to confirm that the ICR mouse colony was free of common murine pathogens especially free of mouse parvovirus. Blood, mesenteric lymph node, spleen and feces were collected. Blood was used for serological testing and tissues were used for PCR evaluation. Serology results indicated that this mouse had no antibodies to the following pathogens: mouse parvovirus, mouse hepatitis virus, murine norovirus, Theiler's encephalomyelitis virus, *Mycoplasma pulmonis*, Ectromelia virus, epizootic diarrhea of infant mice virus, lymphocytic choriomeningitis virus, pneumonia virus of mice, reovirus type 3, sendai virus and minute virus of mice. DNA was extracted from spleen, MLN and feces and tested using the MPV PCR assay (Figure 1). No PCR products were obtained, indicating that ICR mice were free of MPV.

The remaining thirteen male mice were divided into two groups: one group having eight male mice was inoculated with mouse parvovirus. The other group of five mice served as uninfected controls. One week after mouse parvovirus inoculation, feces were collected from inoculated mice. DNA was extracted and MPV PCR assay was done to investigate MPV infectious status in these mice (Figure 2). A 258-bp PCR product was found in all fecal DNA from inoculated mice indicating that inoculated ICR male mice were successfully infected with mouse parvovirus.

From three weeks to five weeks post inoculation at the time when the inoculated mice were 7, 8, 9-weeks old, one inoculated mouse was euthanized in each week. Blood, spleen, feces and semen were collected to investigate the infectious status and semen contamination. Serology testing showed that these mice had antibody to MPV. MPV PCR assay showed that mice had mouse parvovirus infection. Semen from 7-weeks-old mouse was PCR negative for MPV, but 8 and 9-weeks-old mice were MPV positive (Figure 3).

2.2. Mouse parvovirus-1 infection and contamination in semen in the IVF mice

Six weeks after Mouse parvovirus inoculation at the time when male mice were 10-weeks-old, *in vitro* fertilization were performed. Five groups of mice were performed for IVF. Two groups of male mice were 10-weeks-old and three groups of male mice were 11-weeks-old when the semen was collected for IVF. In each group, the semen donor was one male mouse inoculated with MPV-1 and one male mouse without inoculation. During IVF, blood, spleen, feces and semen were collected from male mice. Serologic tests indicated that the five inoculated mice had antibody to MPV. PCR of spleen DNA also indicated that these mice were infected with MPV (Figure 4). The five control male mice did not have antibody to MPV as confirmed by serology evaluation and PCR data, indicating they were free of MPV (Figure 4). Evaluation of semen by PCR for MPV showed that only two of the five inoculated mice had MPV contamination in semen. The remaining three mice did not have MPV contamination in semen (Figure 5).

2.3 Results of *in vitro* fertilization (IVF)

When male mice were 10-weeks-old, *in vitro* fertilization was performed by using semen collected from one male mouse inoculated with mouse parvovirus-1 and oocyte-cumulus complex collected from 4-weeks-old uninfected ICR female mice. As a control, IVF was also performed by using semen collected from a 10-weeks-old male ICR mouse without inoculation and oocyte-cumulus complex collected from 4-weeks-old ICR mice. To eliminate the difference from female mice, oocytes collected from one oviduct were used for inoculated male mouse and oocytes collected from the other oviduct from the same mouse were used for the control male mice. IVF was repeated with five groups of mice five times. Figure 7 showed the comparison between these control and infected mice in fertilization rate, cleavage rate and embryonic development rate. Student t-test showed P=0.1072 for fertilization rate, P= 0.2624 for cleavage rate and P= 0.0539 for embryonic development. These data indicated that there is no difference between the inoculated mice and control mice in fertilization rate, cleavage rate and embryonic development.

2.4 Result of MPV in embryos

Four days after IVF, the development of embryos was investigated. Embryos either developed to blastocysts or stopped at any stage before blastocysts or degenerated. Blastocysts and abnormal embryos were collected and separated into two tubes. DNA was extracted and tested by MPV PCR assay. Figure 6 shows PCR data investigating β-actin in embryos which was used as a control to confirm that total DNA was extracted successfully from these embryos. Figure 8 investigated MPV in

DNA extracted from blastocysts and abnormal embryos. PCR data showed that all the embryos including blastocysts and abnormal embryos were free of MPV. These data indicated that Mouse parvovirus inoculated to male mice did not transfer to embryos through *in vitro* fertilization no matter whether embryos developed to blastocysts or abnormal embryos.

3. Discussion

Recent advances in the methodologies for cryopreservation of mouse spermatozoa have opened up a number of opportunities for storage of transgenic mouse strains and rederivation by *in vitro* fertilization. As showed in the first part of this study, microbial pathogens were found in mouse semen, which indicated a potential risk of transmitting microbial pathogens via the cryopreserved semen. Microbial contamination of semen has been reported to cause infection of female recipient (Meyling and Jensen, 1988) and reduce fertilization and embryonic cleavage rates (Guerin *et al*., 1992). Previous studies have clearly demonstrated that IVF-generated livestock embryos are more vulnerable to microbial contamination than *in vivo* derived embryos (Marquant *et al*., 1998; Bielanski *et al*., 1997). Thus, the potential risks of microbial transmission by assisted reproductive technologies (ART), such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) could be higher. Several reasons may account for the increased risk, as follows. (1) The morphology and physiology of *in vitro*-derived embryos differ from *in vivo*-derived embryos (Wright and Ellington, 1996), which may change host-pathogen interactions and allow increased pathogen transmission. (2) IVF and ICSI circumvent the normal host defense system, thus pathogens that would have been eliminated by the host may proliferate and/or survive that ART process (Kim *et al*., 1998). (3) The frequent handling steps associated with ART increase the opportunities for contamination of gametes and embryos.

Considering many embryos are produced *in vitro* through assisted

reproductive technologies, and Mouse parvovirus was the most prevalent pathogen among the five pathogens found in mouse gametes and embryos from our studies in Chapter 1, this study aimed at investigating the effect of Mouse parvovirus infection in male mice on the fertilization rate, cleavage rate, and embryonic development during *in vitro* fertilization and whether Mouse parvovirus infection in male mice was transmitted to embryos. Our hypothesis is that mouse parvovirus transmits to blastocysts derived from *in vitro* fertilization and affects fertilization, cleavage and embryonic development.

First male mice were inoculated with Mouse Parvovirus-1, and serology and PCR tests confirmed that all the inoculated mice were successfully infected with MPV, however, only two mice out of five showed semen contamination in the five male mice used for IVF experiment. Although two inoculated mice euthanized at 8 and 9-weeks-old had MPV contamination in semen, we did not perform IVF until male mice were 10-weeks-old because of the following two reasons: (1) Mice at 7, 8, 9 weeks were too young to produce adequate semen for IVF. (2) In the first part of this study, 10-weeks-old Sencar male mice showed the highest MPV prevalence in mouse semen. Thus in the experimental design we planned to initiate IVF when male mice were 10-weeks-old. Several factors may cause low prevalence of MPV contamination in semen of the experimental male mice. First, different strains of mice (Sencar and ICR mouse), naturally infected and inoculated mice, may have different responses to virus infection and show different time periods for contamination of semen. It is likely that the inoculated ICR mouse gradually stopped shedding virus in semen after ten

weeks. Another reason could be the dose we inoculated to the male mice was not strong enough to cause the semen contamination.

None of the embryos were contaminated by MPV as confirmed by MPV PCR assay, different from what was originally expected. Fertilization rate, cleavage rate, and embryonic development were not affected by Mouse parvovirus infection in male mice, also different from what was expected (Figure 8). One reason could be the low prevalence of contamination in semen which only two out of five mice had semen contamination. Although the other three male mice had infection, their semen was free of Mouse parvovirus contamination. It was also possible that Mouse parvovirus was eliminated during the washings. There were three washes when oocyte-cumulus complex was added to the *in vitro* fertilization medium and three washes when fertilized oocyte was transferred to culture medium. If the washings reduced and/or eliminated the viruses, we may conclude that Mouse parvovirus contamination in mouse semen was less likely to transmit the contamination to the embryos produced by *in vitro* fertilization and affect fertilization, cleavage and embryonic development if appropriate washes were performed. Additional experiments are needed to further investigate this phenomenon and determine the reason for low semen contamination prevalence.
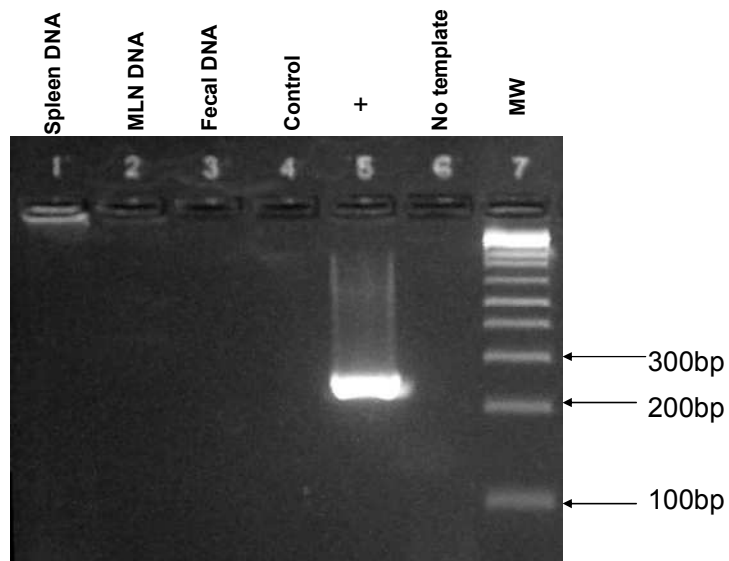
Figure 1. Agarose gel showing MPV specific PCR products obtained after amplification of spleen, MLN and fecal DNA from ICR male mouse before MPV-1 inoculation. Lane 1 was DNA from spleen. Lane 2 was DNA from MLN. Lane 3 was DNA from feces. Lane 4 was control for reagents used in DNA extraction. Lane 5 was positive control contains MPV DNA. Lane 6 was no template negative control. Lane 7 was molecular weight marker. Data showed that ICR mice were free of MPV.
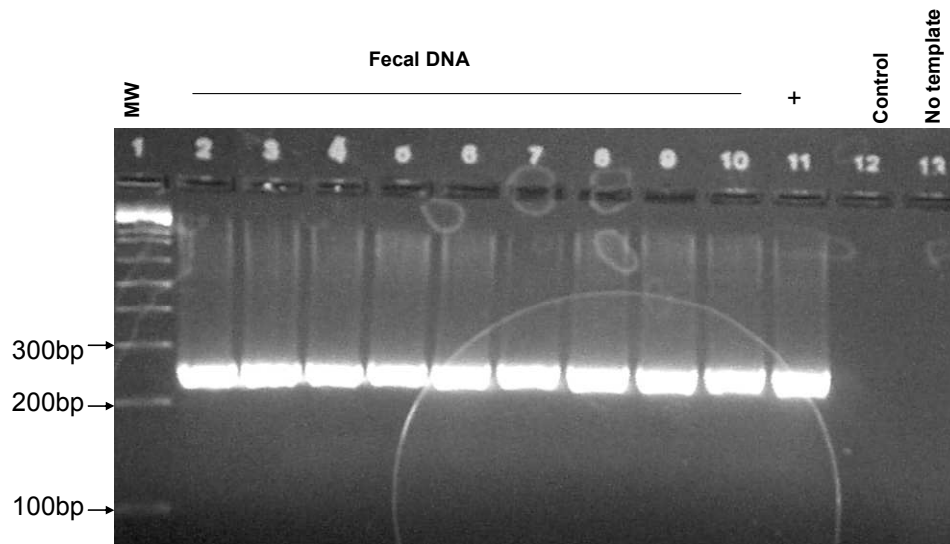
Figure 2. Agarose gel showing MPV-specific PCR products obtained after amplification of fecal DNA from inoculated male mice one week post inoculation (lanes 2 to 10). Lane 1 was molecular marker, and lane 11 was positive control. Lane 12 was control for reagents used in DNA extraction. Lane 13 was the PCR no template negative control. Data showed that ICR male mice were successfully infected with Mouse parvovirus.
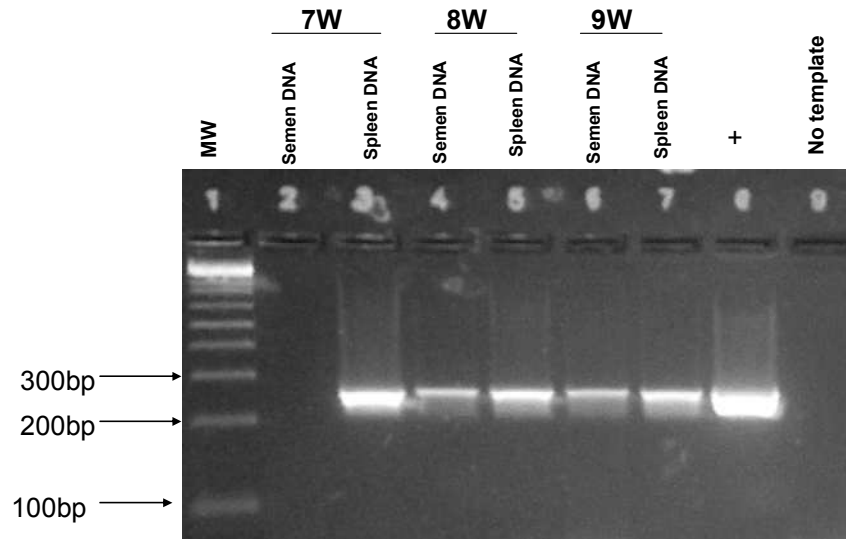
Figure 3. Agarose gel showing MPV-specific PCR products obtained after amplification of semen and spleen DNA from 3, 4, 5-weeks post inoculation (inoculated male mice were 7, 8, 9-weeks-old). Lane 1 was molecular marker. Lane 2, 4, 6 were DNA from semen collected from 3, 4, 5-weeks post inoculation mice respectively. Lane 3, 5, 7 were DNA from spleen collected from 3, 4, 5-weeks post inoculated mice respectively. Lane 8 was positive control. Lane 9 was no template negative control. Data showed that male mice were infected with MPV, but only 4, and 5-weeks post inoculated mice had MPV contamination in semen.
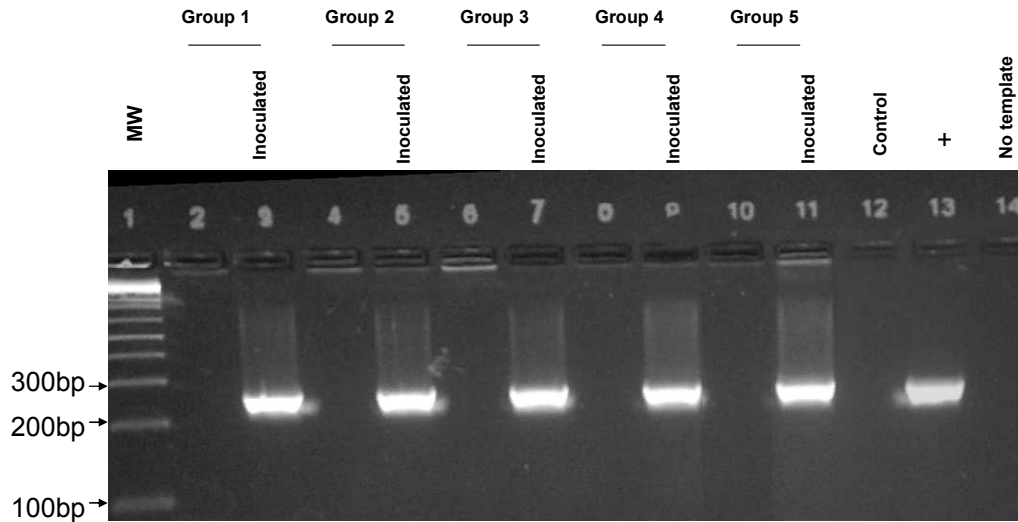
Figure 4.   Agarose gel showing MPV-specific PCR products obtained after amplification of spleen DNA from inoculated and control male mice for IVF. Lane 1 was molecular marker. Lane 2, 4, 6, 8, 10 were DNA from control male mice without MPV inoculation in group 1, 2, 3, 4, 5 respectively. Lane 3, 5, 7, 9, 11 were DNA from inoculated male mice in group 1, 2, 3, 4, 5 respectively. Lane 12 was control for reagents used in DNA extraction. Lane 13 was positive control. Lane 14 was PCR no template negative control. Data showed that inoculated male mice were infected with MPV. Control mice were free of MPV.
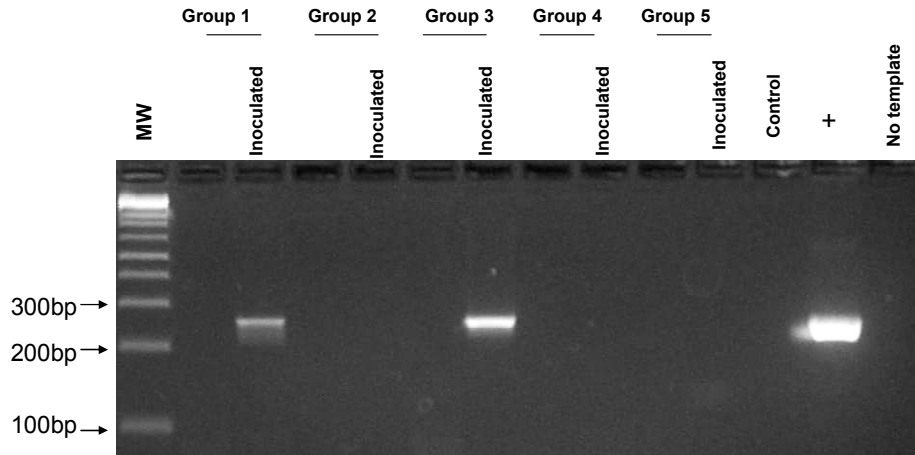
Figure 5. Agarose gel showing MPV-specific PCR products obtained after amplification of semen DNA collected from inoculated and control male mice for IVF. Lane 1 was molecular marker. Lane 2, 4, 6, 8, 10 were semen DNA extracted from male mice without inoculation in group 1, 2, 3, 4 and 5 respectively. Lane 3, 5, 7, 9, 11 were semen DNA extracted from inoculated male mice in group 1, 2, 3, 4 and 5 respectively. Lane 12 was control for reagents used for DNA extraction. Lane 13 was positive control. Lane 14 was PCR no template negative control. Data showed that inoculated mice in group 1 and 3 had MPV contamination in semen. None of control mice had MPV contamination in semen.
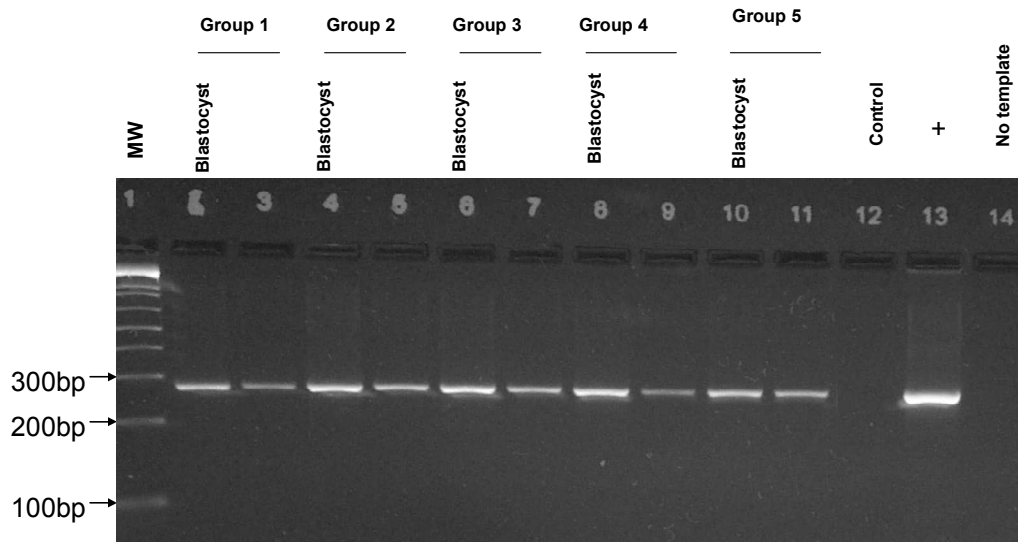
Figure 6. Agarose gel showing β-actin-specific PCR products obtained after amplification of embryo DNA. Lane 1 is molecular marker. Lane 2, 4, 6, 8, 10 were DNA from blastocysts of group 1, 2, 3, 4, 5 respectively. Lane 3, 5, 7, 9, 11 were DNA from abnormal embryos of group 1, 2, 3, 4, 5 respectively. Lane 12 was control for reagents used for DNA extraction. Lane 13 was positive control. Lane 14 was PCR no template negative control. Data showed that DNA was successfully extracted from blastocysts and abnormal embryos.
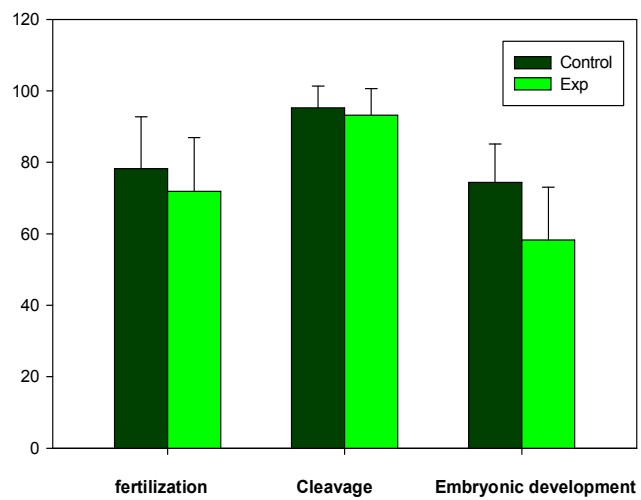
Figure 7 Comparison of fertilization rate, cleavage rate and embryonic development between control mice and inoculated mice.
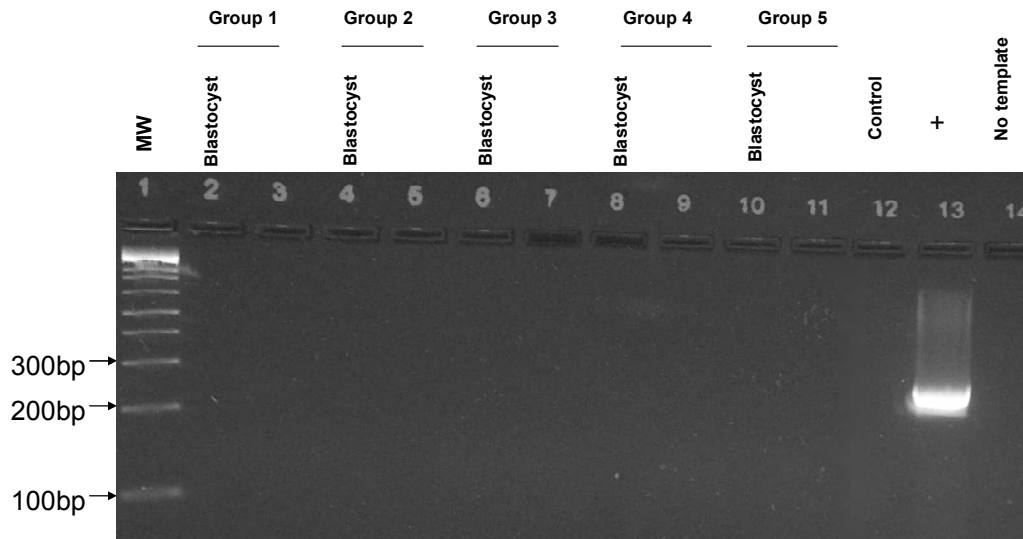
Figure 8 Agarose gel showing MPV specific PCR products obtained after amplification of embryo DNA. Lane 1 is the molecular marker. Lane 2, 4, 6, 8, 10 were DNA from blastocysts of group 1, 2, 3, 4, 5 respectively. Lane 3, 5, 7, 9, 11 were DNA from abnormal embryos of group 1, 2, 3, 4, 5 respectively. Lane 12 was control for reagents used for DNA extraction. Lane 13 was positive control. Lane 14 was no template negative control. Data showed that none of blastocysts and abnormal embryos had MPV contamination.

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