# Risk Assessment of Mouse Hepatitis Virus Infection via In Vitro Fertilization and Embryo Transfer by the Use of Zona-Intact and Laser-Microdissected Oocytes

**ARTICLE** *in* BIOLOGY OF REPRODUCTION · MARCH 2006

Impact Factor: 3.32 · DOI: 10.1095/biolreprod.105.045112 · Source: PubMed



**7 AUTHORS**, INCLUDING:

Dominika Daria Peters
Quintiles GmbH
**5** PUBLICATIONS **61** CITATIONS
SEE PROFILE

Auke Boersma
University of Veterinary Medicine, Vienna
**20** PUBLICATIONS **317** CITATIONS
SEE PROFILE

Jörg Schmidt
Helmholtz Zentrum München
**125** PUBLICATIONS **2,234** CITATIONS
SEE PROFILE

BIOLOGY OF REPRODUCTION **74,** 246–252 (2006)
Published online before print 12 October 2005.
DOI 10.1095/biolreprod.105.045112

# Risk Assessment of Mouse Hepatitis Virus Infection via In Vitro Fertilization and Embryo Transfer by the Use of Zona-Intact and Laser-Microdissected Oocytes[1]

**Dominika D. Peters,[3] Susan Marschall,[3] Esther Mahabir,[4] Auke Boersma,[3] Ulrich Heinzmann,[5] Jörg Schmidt,[4] and Martin Hrabé de Angelis[2,3]**

*Institute of Experimental Genetics,[3] Department of Comparative Medicine,[4] Institute of Pathology,[5] GSF - National Research Center for Environment and Health, D-85764 Neuherberg, Germany*

## ABSTRACT

**The aim of this study was to estimate the risk of mouse hepatitis virus (MHV) transmission by the in vitro fertilization and embryo transfer (IVF-ET) procedure. In addition, resistance to infection of zona-intact and laser-microdissected oocytes was compared. For this purpose, infectious mouse hepatitis virus, a common viral pathogen in mouse facilities, was used. Oocytes having an intact or laser-microdissected zona pellucida were incubated for fertilization in media containing MHV-A59 and resulting embryos were transferred to the oviduct of specific pathogen-free (SPF) Swiss recipients. The oocytes were divided into three experimental groups: 1) zona-intact oocytes continuously exposed to MHV in fertilization (HTF), culture (KSOM), and embryo transfer (M2) media; 2) zona-intact oocytes exposed to MHV in HTF medium and transferred after a standard washing procedure with virus-free KSOM and M2; and 3) laser-microdissected oocytes exposed to MHV in HTF medium and transferred after a standard washing procedure with virus-free KSOM and M2. Respective serum samples of embryo recipients and their offspring were tested for MHV antibodies using ELISA. In experiment 1, 10 out of 14 embryo recipients seroconverted to MHV and only their offspring (8 of 19) received maternal antibodies. In experiments 2 and 3, MHV antibodies were detected neither in the recipients nor in the offspring. These results indicate, for the first time, that even if the zona pellucida is partially disrupted by laser microdissection, the transmission of MHV-A59 can be avoided by correctly performed washing steps in the IVF-ET procedure.**

*assisted reproductive technology, in vitro fertilization, MHV infection*

## INTRODUCTION

Mice with standardized health status, according to the Federation of European Laboratory Animal Science Association (FELASA) recommendations for health monitoring of laboratory animals [1, 2], are crucial prerequisites for the reproducibility of animal experiments. Due to the fact that subclinical infections can have an impact on the physiological reaction of mice under experimental conditions, the demand for such animals in current research is rapidly increasing. Specific pathogen-free (SPF) mice are generally obtained by hysterectomy or by embryo transfer [3–7]. Additionally, embryo transfer is a well-established procedure used to recover in vivo- and in vitro-produced embryos stored in frozen embryo banks [8] or for recovery of frozen sperm samples [9, 10].

Ever since large-scale mouse mutagenesis projects have been initiated [11–13], numerous mouse lines have been generated and cryopreserved [10, 14, 15]. Cryopreservation of mouse germplasm generally provides means for long-term storage of valuable mouse lines and facilitates the management of animal facilities. Moreover, distributing frozen material instead of live animals reduces the risk of pathogen transmission and contributes to animal welfare.

In vitro fertilization has been frequently used to improve defects in the reproductive ability of mutant or aged mice [10, 14–19]. Inbred mouse strains have defined reproductive parameters, which are characteristic of the strain. Differences in the ability of spermatozoa to survive cryopreservation and to fertilize oocytes of the same genetic background were described in several studies [15, 17, 20]. Successful sperm cryopreservation is defined by the recovery of live offspring from frozen-thawed spermatozoa. To increase the efficiency of fertilization, the routinely used rederivation methods have to be enhanced by assisted reproductive technologies, such as partial zona dissection (PZD) [21–23], zona incision by piezomanipulator (ZIP) [24, 25], subzonal insemination (SUZI) [26], intracytoplasmic sperm injection (ICSI) [27, 28], and zona microdissection (ZD) [26, 29–32]. An intact zona pellucida was shown to act as a barrier to protect embryos from pathogen transmission [4, 7, 33–35]. Therefore, assisted reproductive methods with chemically or mechanically manipulated zona pellucida need to be investigated with regard to possible pathogen transmission.

Methods for culture and washing of preimplantation mouse embryos have been established for many years. For example, a mouse rederivation program for in vivo-produced embryos that is effective in eliminating viral, bacterial, or parasitic infections has been reported [4, 7, 33, 34]. Using in vitro-produced embryos, elimination of mouse hepatitis virus (MHV) and *Pasteurella pneumotropica* (P.p.) from contaminated sperm and oocyte donors was reported [36].

The aim of the present study was to investigate whether the presence of MHV-A59 in the in vitro fertilization (IVF) leads to infection of embryos and consequently to infection of recipients and their progeny. For this reason, embryos were exposed to MHV-A59 and transferred to SPF pseudopregnant recipients. Antibody production to MHV was monitored by ELISA in recipients and pups. In addition, resistance to infection of zona-intact and laser-microdissected oocytes was investigated.

## MATERIALS AND METHODS

### *Animals*

Inbred C3HeB/FeJ mice served as sperm (12 wk old) and oocyte (21–28 day old) donors. Outbred Swiss female mice (6–8 wk old) were used as

[1]Supported by the European Commission under FP6, by EMMAinf (506455) to MHdA and by the grant of the National Genome Research Network, NGFN2 (01GR0103) to MHdA.
[2]Correspondence: FAX: 0049 89 3187 3500; e-mail: hrabe@gsf.de

Received: 1 July 2005.
First decision: 29 July 2005.
Accepted: 5 October 2005.

ISSN: 0006-3363. http://www.biolreprod.org

pseudopregnant embryo transfer recipients. Both mouse strains were bred at the GSF-National Research Center for Environment and Health animal facility and were tested for microorganisms according to the FELASA recommendations for the health monitoring of mice [2]. Health monitoring was performed every 12 wk using 12-wk-old SPFsentinel mice. The husbandry conditions were as follows: room temperature 20–24°C, air humidity 50–60%, 20 air changes per hour, lighting regimen on a 12L:12D cycle. Wood shavings (Altromin; Lage, Germany) were applied as bedding material. Mice were fed with standardized mouse diet (1314 Altromin) and drinking water ad libitum.

The oocyte donors were shipped to the SPF barrier unit in filter-topped boxes (PTS Pharma-Techn.-Service, Sasbach) 1 wk before the start of the experiments. The in vitro fertilization and embryo transfer (IVF-ET) procedure was performed in an embryo-manipulation laboratory in the barrier unit. Upon arrival, each embryo recipient was placed into a separate autoclaved individually ventilated cage (Ventirack; Biozone, U.K.) with wood shavings, food, and filtered (0.2 μm) water ad libitum. All animal manipulations were performed in a class-II laminar-flow biology safety cabinet (Heraeus Instruments, Germany). The animal experiments were approved by the Government of Bavaria in Germany.

## Virus Stock

The virus stock MHV-A59 (VR-764), the cell line NCTC (clone 1469, CCL-9.1), and the murine fibroblasts L-929 (CCL-1) were obtained from American Type Culture Collection (ATCC; Manassas, VA). Propagation of the virus stock was performed in 75-$cm^2$ cell culture flasks (Costar flasks; Corning B.V., Schiphol-Rijk, The Netherlands) at 37°C using 5% $CO_2$ and 100% atmospheric humidity. The viruses were propagated in NCTC-1469 cells. The supernatants were filtered and frozen in aliquots at −80°C. NCTC-1469 and L-929 cells were cultured in Dulbecco minimum essential medium (DMEM; Biochrom AG, Berlin, Germany) containing 10% heat-inactivated fetal calf serum (FCS; Biochrom/Seromed, Berlin, Germany). The tissue-culture infectious dose ($TCID_{50}$/ml) was determined in L-929 cells and calculated according to the Spearman-Kaerber method [37, 38].

## Transfer of Virus Suspension to the Oviduct of Pseudopregnant Recipients

To determine the virus dose required to induce seroconversion of embryo recipients, different dilutions of MHV-A59 suspension containing $10^9$, $10^8$, $10^7$, and $10^5$ $TCID_{50}$/ml were inoculated into the oviducts of 6–8-wk-old pseudopregnant embryo recipients. The volume of inoculum was 0.05 μl per mouse and corresponded to that used in routine embryo transfer. Control mice were inoculated with 0.05 μl of 0.9% NaCl or M2 (Sigma-Aldrich, Taufkirchen, Germany). Blood samples were taken on Days 14, 21, 28, and 42 after inoculation from each mouse by lateral tail vein (Caudal v.) puncture and sera were tested as described below.

## Serological Analysis

The serum samples were tested at The Microbiology Laboratories (TML) in North Harrow, U.K. Prior to the test, the serum samples were inactivated by heating to 56°C for 30 min and diluted 1:10 in PBS (Oxoid, Hants, U.K.) containing 0.05% Tween 20 (R&L Slaughter, Essex, U.K.). Sera were tested for specific antibodies to MHV by use of an enzyme-linked immunosorbent assay (ELISA) using nonviral control and viral antigens and negative and positive serum (Churchill; Applied Biotechnology Ltd. Cambridgeshire, U.K.) [39]. The control sera undergo quality-control tests (ISO accreditation). The optical density (OD) was measured with the Multiscan ELISA plate reader (Thermo Life Sciences, Hampshire, U.K.). Serum was considered to be equivocal low positive between 0.600 and 0.799 OD and positive when OD values exceeded 0.799 OD.

## Collection, Freezing, and Thawing of Sperm Samples

Three-month-old C3HeB/FeJ males were killed by cervical dislocation. Both caudae epididymides and vasa deferentia were removed and placed in 220 μl of cryoprotectant solution (CPA) containing 3% skim milk and 18% raffinose. The cryoprotectant solution was prepared according to the method described by Nakagata and Takeshima [20]. The tissues were cut with sterile injection needles (B. Braun, Melsungen, Germany) and the sperm were allowed to disperse for 10 min at 37°C and 5% $CO_2$. The dish was gently shaken until the sperm suspension was homogeneous. The sperm was loaded in 15-μl aliquots to 10 straws (0.25 ml; Minitüb, Tiefenbach, Germany). Straws were heat sealed and placed in liquid nitrogen vapor (≈ −120°C). Ten minutes later, the straws were stored in liquid nitrogen. For thawing, one straw was put in a water bath (37°C) for 2 min and 4 μl of thawed sperm samples was placed in 200-μl drops of human tubal fluid (HTF) medium under mineral oil (embryo tested; Sigma-Aldrich). The HTF drops with thawed spermatozoa were incubated at 37°C and 5% $CO_2$ and used for in vitro fertilization. The sperm quality was determined using a Hamilton Thorn IVOS computerized semen analyzer (Hamilton Thorn, Beverly, MA) as described [17]. The HTF medium was prepared from reagent-grade chemicals (Sigma-Aldrich) as described by Quinn et al. [40].

## Laser Microdissection of the Zona Pellucida

The VSL-337-ND-S nitrogen laser, with an output of 337 nm (PALM Microlaser Technologies, Bernried, Germany), was used for microdissection of the zona pellucida. The laser microbeam system was coupled with an inverted microscope (Carl Zeiss, Oberkochen, Germany) and the image of the oocytes was transmitted by a video camera (Sony Corporation, Tokyo, Japan) to the monitor system. The laser UV beam was focused on a glass slide (cat. no. 440–110; PALM Microlaser Technologies, Germany) covered with a 1.35-μm-thick membrane and used for microdissection of the zona pellucida. Aliquots of 100 μl each of PBS-BSA medium were placed into laser microbeam dishes (PetriPALM50 hydrophilic; PALM Microlaser Technologies) under mineral oil. The oocytes were denuded of cumulus cells by washing in PBS-BSA medium (Sigma-Aldrich) containing 600 μg/ml working concentration of bovine testes hyaluronidase (Sigma-Aldrich). Twenty to 50 cumulus-free metaphase II oocytes were distributed into each drop of the PBS-BSA medium, and the petriPALM dishes were fixed on the x, y, z microscope stage. The 40× LD Achroplan lens was focused on the zona pellucida of the oocytes and cuts of about 5 μm width and 20 μm length were performed. The cutting points were located, if possible, opposite to the polar bodies of the oocytes without complete penetration of the zona pellucida. Under visual control, the zona pellucida was approximately, but not completely, penetrated. Oocytes with completely disrupted zona pellucida were discarded. The success of the microdissection procedure was verified on the computer screen. After laser microdissection, the oocytes were placed into fertilization dishes with capacitated spermatozoa.

## Electron Microscopy

To show the extent of damage on the zona pellucida in more detail, a small number of microdissected oocytes were examined using scanning electron microscopy, SEM (Fig. 1). The oocytes for scanning electron microscopic examination were washed in PBS (pH of 7.4) and prefixed in 4% paraformaldehyde in PBS overnight. The samples were dehydrated in a graded series of ethanol, critical-point dried, and sputter coated (K575 EMITECH LTD, Ashford, England) with 5 nm platinum. Coated samples were examined in a field emission scanning electron microscope (Jeol JSM-6300F, Tokyo, Japan) with accelerating voltages of 5 kV in secondary electron mode.

## In Vitro Fertilization and Washing Procedure

Twenty-one to 28-day-old inbred C3HeB/FeJ female mice were superovulated by i.p. injection of 5 IU of eCG (Intergonan 1000; Intervet, Unterschleißheim, Germany) followed by 5 IU of hCG (Ovogest 1500; Intervet) 48 h later. Thirteen to 14 h after injection with hCG, mice were killed by cervical dislocation and the oviducts were removed. The cumulus-oocyte complexes were isolated from oviducts in a 500-μl HTF drop under mineral oil and added to fertilization dishes (oocytes from 3–5 females per each fertilization dish) with preincubated sperm samples. The MHV-A59 suspension ($10^8TCID_{50}$ per ml) was used for the contamination of IVF media at a dilution of 1:10. After a 5-h incubation (5% $CO_2$, 37°C, and 95% humidity), the oocytes were washed four times through four medium changes (100 μl each) of KSOM (Powdered Media Kit; Metachem Diagnostics Ltd., Northampton, U.K.) and incubated overnight in the fifth 100-μl drop of KSOM under mineral oil. Twenty-four hours after the start of the IVF, the two-cell embryos were washed through four medium drops (100 μl each) of M2 medium and transferred to Day 0.5 pseudopregnant recipients [18] in the fifth 100-μl drop of M2. Altogether, the oocytes/embryos were washed 10 times through virus-free KSOM and M2 medium. A new sterile glass capillary was used between each IVF dish. The dilution factor was approximately 1:100 per washing step. The maximum number of oocytes washed at the same time was 100. The cleavage rate is defined as the percentage of morphologically intact two-cell embryos having blastomeres of approximately the same size from the total number of oocytes placed in the fertilization dishes.

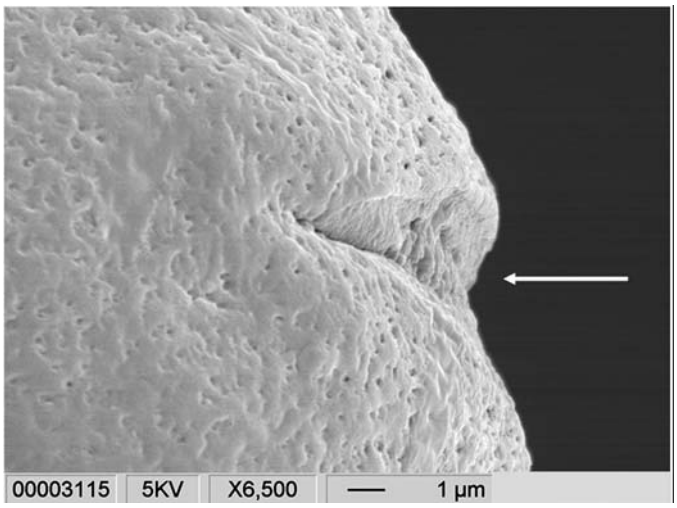


FIG. 1. Arrow indicates laser-microdissected sites of zona pellucida under scanning electron microscopy. Original magnification ×6500.

### Embryo Transfer

Two-cell embryos from each experimental group were transferred under sterile surgical procedures into the oviduct of pseudopregnant Swiss mice. Each embryo recipient received 20 two-cell embryos divided between the oviducts. The diameter and the length of the medium containing embryos in the glass capillary were measured with an integrated lineal of PALM Robo Combisystem. The average volume of the medium (0.05 µl) was defined as $V = 3.14 \times r^2 \times h$, where $V$ is the volume, $r$ is the mean radius (55 ± 3.74 µm) of the glass capillary, $h$ is the mean length (0.023 ± 0.0075 µm) of the medium containing embryos. After embryo transfer, the recipients were housed separately in autoclaved individually ventilated cages. Surgery was performed under general anesthesia with appropriate analgesic support as described by [18].

### Virological Examination of Remaining Media and Washing Fluids

After completion of the washing steps, the remaining fluids of the HTF, KSOM, and M2 media were placed into sterile 1-ml Eppendorf tubes and stored at −80°C. These samples were tested for the presence of MHV using RT-PCR according to the method of Taylor and Copley [41] and for virus infectivity by titration on the cell line L-929.

### Experimental Design

To determine whether the presence of MHV in the IVF and culture system leads to an immune response of the embryo recipients, the oocytes were divided into the following experimental groups.

*Experimental group 1.* Zona-intact C3HeB/FeJ oocytes were continuously exposed to $10^8$ $TCID_{50}$/ml of MHV-A59 suspension in HTF, KSOM, and M2 media during fertilization, culture, and embryo transfer, respectively, and the resulting embryos were transferred to the oviducts of SPF recipients.

*Experimental group 2.* Zona-intact C3HeB/FeJ oocytes were exposed to $10^8$$TCID_{50}$/ml of MHV-A59 suspension in HTF medium only during fertilization, washed 10 times with virus-free KSOM and M2 medium, and the resulting two-cell embryos were transferred to the oviducts of SPF recipients.

*Experimental group 3.* Laser-microdissected C3HeB/FeJ oocytes were exposed to $10^8$$TCID_{50}$/ml of MHV-A59 suspension in HTF medium only during fertilization, washed 10 times with virus-free KSOM and M2 medium, and the resulting embryos were transferred to the oviducts of SPF recipients.

All experiments were performed five times and the serum samples of the recipients and offspring were tested for MHV antibodies. Two criteria were used to determine whether the rederived mice were MHV free: 1) embryo recipients were seronegative for MHV on Days 21, 28, and 42 after embryo transfer and 2) offspring were seronegative to MHV on Days 21 and 42 after birth.

TABLE 1. Immune response of embryo transfer recipients after oviduct transfer of MHV-A59.

| | | Seroconversion to MHV-A59 (number of positive/total animals tested)[a] | | | |
|---|---|---|---|---|---|
| Virus titer ($TCID_{50}$/ml) | Virus dose/mouse ($TCID_{50}$) | Day 14 | Day 21 | Day 28 | Mortality |
| $10^9$ | $5x10^4$ | 2/6 | 5/6 | 5/6 | 1/6[b] |
| $10^8$ | $5x10^3$ | 4/6 | 4/6 | 4/6 | 1/6[b] |
| $10^7$ | $5x10^2$ | 0/5 | 0/5 | 0/5 | 0/5 |
| $10^5$ | $5x10^0$ | 0/5 | 0/5 | 0/5 | 0/5 |
| NaCl | 0 | 0/2 | 0/2 | 0/2 | 0/2 |
| M2 | 0 | 0/2 | 0/2 | 0/2 | 0/2 |
| DMEM | 0 | 0/2 | 0/2 | 0/2 | 0/2 |

[a] Mean OD < 0.600 = negative; 0.600 – 0.799 = equivocal low positive; > 0.799 = positive.
[b] Mouse died due to infection.

Furthermore, the effect of MHV-A59 on the cleavage rate and subsequent embryonic development of mice was determined.

### Ethical Review Procedure

All experimental protocols were approved by the local ethical review committee with respect to the care and use of laboratory animals for scientific purposes.

### Statistical Analysis

The statistical analysis of the immune response of embryo recipients was applied among experimental groups using Bernoulli pattern for the number of MHV antibody-positive and -negative embryo recipients. The statistical analysis of the cleavage rate and offspring recovery was evaluated by the chi-square test. Values with $P < 0.001$ were considered statistically different.

## RESULTS

### Immune Response of Embryo Recipients after Oviduct Transfer of Virus Suspensions

The immune response of Day 0.5 pseudopregnant recipient mice after oviduct transfer with different dilutions of MHV-A59 suspension is summarized in Table 1. After inoculation of $5 \times 10^0$ $TCID_{50}$ and $5 \times 10^2$ $TCID_{50}$ MHV-A59 per mouse or with 0.9% NaCl, M2, and DMEM medium as a negative control, the recipient mice showed no antibody production against MHV on Days 14, 21, and 28 after inoculation (OD < 0.600). Embryo recipients that received $5 \times 10^4$ or $5 \times 10^3$ $TCID_{50}$ of MHV-A59 (corresponds to $10^9$ $TCID_{50}$ and $10^8$ $TCID_{50}$ of MHV-A59 per ml suspension) produced antibodies to MHV on Days 14, 21, and 28 after inoculation. Inoculation with $5 \times 10^4$ $TCID_{50}$ of MHV-A59 caused seroconversion in 2 of 6 pseudopregnant recipients on Day 14 (mean OD = 0.734 ± 0.245) and in 5 of 6 recipients on Day 21 (mean OD = 0.849 ± 0.175) and Day 28 (mean 1.044 ± 0.326) after inoculation. Inoculation with $5 \times 10^3$ $TCID_{50}$ of MHV-A59 caused seroconversion in 4 of 6 pseudopregnant recipients on Day 14 (mean OD = 0.713 ± 0.088), Day 21 (mean OD = 0.862 ± 0.145), and Day 28 (mean OD = 1.265 ± 0.105) after inoculation.

In consideration of this data, a virus titer of $10^8$ $TCID_{50}$ per ml was used in all successive experiments.

### Experimental Groups 1–3

Continuous presence of MHV-A59 in fertilization, culture, and embryo transfer media used for IVF in experimental group 1

TABLE 2. Immune response of embryo transfer recipients after oviduct transfer of zona-intact and laser-manipulated embryos exposed to MHV-A59.

| Experimental groups | Seroconversion to MHV-A59 (number of positive/total animals tested)[a] | | | Mortality |
|---|---|---|---|---|
| | Day 21 | Day 28 | Day 42 | |
| Group 1 HTF, KSOM, M2 with MHV | 9/14 | 10/14 | 10/14[c] | 1/14[b] |
| Group 2 HTF with MHV | 0/12 | 0/12 | 0/12[d] | 0/12 |
| Group 3 HTF with MHV + laser | 0/14 | 0/14 | 0/14[d] | 0/14 |
| Control (transfer of M2) | 0/8 | 0/8 | 0/8[d] | 0/8 |

[a] Mean OD $< 0.600$ = negative; 0.600 – 0.799 = equivocal low positive; $> 0.799$ = positive.
[b] Mouse died due to infection.
[c,d] Values with different superscripts within a column are significantly different ($P < 0.001$; Bernoulli pattern).

caused seroconversion in 9 of 14 pseudopregnant recipients on Day 21 (mean OD = 1.126 ± 0.276) and in 10 of 14 recipients on Day 28 (mean OD = 1.330 ± 0.321) and Day 42 (mean OD = 1.325 ± 0.524) after embryo transfer (Table 2). Only offspring (8 out of 19) descending from 10 seropositive tested for MHV antibodies embryo recipients received maternal antibodies to MHV (Table 3) on Day 21 after birth (mean OD = 1.945 ± 0.209). Progeny (11 out of 19) descending from recipient females without seroconversion did not show MHV antibodies on Day 21 after birth (OD $< 0.600$). Compared with experimental group 1, no embryo recipient from experimental groups 2 and 3 (Table 2) showed antibody production to MHV on Days 21, 28, and 42 after embryo transfer (OD $< 0.600$). Similarly, the pups born in experimental groups 2 and 3 (Table 3) had no antibodies to MHV on Day 21 after birth (OD $< 0.600$).

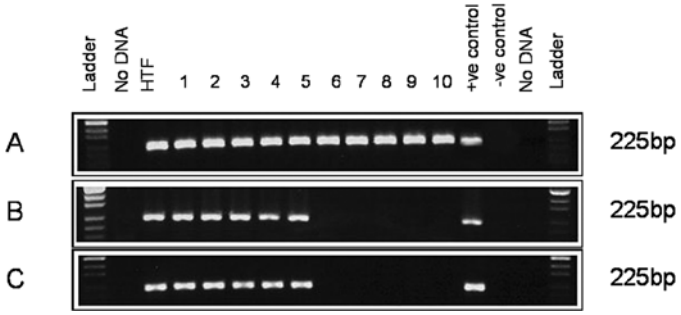


FIG. 2. MHV RT-PCR analysis using remaining media of 10 washing steps within experimental groups 1–3. A) Experimental group 1 (HTF, KSOM, M2 with MHV). B) Experimental group 2 (HTF with MHV). C) Experimental group 3 (HTF with MHV + laser). Lanes 1–5: washing steps with KSOM; lanes 6–10: washing steps with M2.

### *Virological Examination of Remaining Media and Washing Fluids*

In experimental group 1, PCR examination of the remaining fluids from 10 washing steps during the IVF-ET procedure confirmed the presence of MHV-A59 (Fig. 2A). In the remaining fluids of experimental groups 2 and 3, MHV-A59 was detected in the HTF medium and in the washing steps 1–5 (HTF and KSOM) but not in the washing steps 6–10 (M2) (Fig. 2, B and C).

### *In Vitro Cleavage Rates and Birth Rates*

Significant differences in the cleavage rate were observed between group 2 and the control IVF (39.9 vs. 51.1%, $P < 0.001$, Table 3). The percentage of pregnant recipients in the experimental groups 1 and 2 and control IVF were 69.2%, 75.0%, and 87.5%, respectively. The percentage of live young was 13.8%, 15.0%, and 16.1%, respectively. Significant differences were not detected among these groups ($P > 0.05$, Table 3).

TABLE 3. Cleavage rate and recovery of C3HeB/FeJ offspring from zona-intact and laser-manipulated IVF oocytes exposed to MHV-A59.

| Experimental groups[a] | No. of oocytes used[b] | No. of two-cells[b] (%) | No. of pregnant/ recipient females[b] (%) | No. of 2-cells transferred[b] | No. of live young[b,c] (%) | Immune response[d] | |
|---|---|---|---|---|---|---|---|
| | | | | | | No. of MHV positive embryo recipients/ no. of embryo recipients used | No. of MHV positive pups/ no. of pups born |
| Group 1 HTF, KSOM, M2 with MHV-A59 | 176.0 ± 37.5 | 75.8 ± 36.6 (43.0)[e] | 1.8 ± 0.8/2.6 ± 0.9 (69.2)[e] | 36.0 ± 16.7 | 5.0 ± 5.7 (13.8)[e] | 10/14 | 8/19 |
| Group 2 HTF with MHV-A59 | 142.6 ± 52.9 | 57.0 ± 16.4 (39.9)[f] | 1.8 ± 1.1/2.4 ± 0.9 (75.0)[e] | 36.0 ± 21.9 | 5.4 ± 4.3 (15.0)[e] | 0/12 | 0/26 |
| Group 3 HTF with MHV-A59+laser | 187.6 ± 56.1 | 75.0 ± 19.5 (39.9)[f] | 1.4 ± 0.9/2.8 ± 0.4 (50.0)[e] | 56.0 ± 8.9 | 3.8 ± 3.3 (6.8)[e] | 0/14 | 0/10 |
| Control (groups 1, 2) HTF, KSOM, M2 virus-free | 158.3 ± 145.3 | 81.0 ± 63.5 (51.1)[e] | 2.3 ± 1.5/2.6 ± 1.5 (87.5)[e] | 53.3 ± 30.5 | 8.6 ± 5.5 (16.1)[e] | 0/8 | 0/26 |
| Control (group 3) HTF, KSOM, M2 virus-free +laser | 47.2 ± 8.8 | 34.5 ± 7.7 (73.0)[e] | 1.5 ± 2.1/3 ± 0 (50.0)[e] | 60 ± 0 | 2.0 ± 2.8 (3.3)[e] | nt | nt |

[a] In each experimental group, 5 IVF's were performed and the mean value of oocytes used per IVF was shown; nt, not tested.
[b] Data represent mean ± SD.
[c] Total number of live-born offspring; the percentages are expressed as the number of mice obtained from the total number of embryos transferred.
[d] Specific antibodies to MHV-A59 (embryo recipients sera tested on day 21, 28, and 42 after embryo transfer; pups sera tested on day 21 after birth).
[e,f] Values with different superscripts within a column are significantly different in comparison to appropriate control IVF ($P < 0.001$; chi square test).

The cleavage rates of the experimental group 3 and control IVF (Table 3) was 39.9% and 73.0%, respectively ($P < 0.001$). The percentage of pregnant recipients was 50% in both groups and the percentage of live offspring was 6.8% and 3.3%, respectively ($P > 0.05$).

## DISCUSSION

Because the importance of SPF mice for the production of reliable scientific data has been recognized, different procedures have been applied to eliminate pathogens from infected mouse stock. These procedures include hysterectomy, IVF, and ET. Using the routine IVF-ET procedure in our laboratory, it was shown that transmission of MHV-A59 was avoided by repetitive washing steps, even when the zona pellucida was partially disrupted by laser microdissection.

The transfer of different dilutions of MHV-A59 suspension containing $10^9$, $10^8$, $10^7$, and $10^5$ $TCID_{50}$ per ml to the oviduct of pseudopregnant recipients allowed determination of $5 \times 10^3 TCID_{50}$ of MHV-A59 as the critical virus dose per mouse, which caused seroconversion of 8-wk-old Swiss recipients. On the other hand, it was shown that a small volume of approximately 0.05 µl medium per mouse, which was used in a routinely performed oviduct transfer, led to infection of the recipient mice only if the virus titer was at least $10^8$ $TCID_{50}$ of MHV-A59 per ml.

Several studies used embryos produced in vivo or in vitro from mice that harbored different viral or bacterial pathogens, including Sendai virus, MHV, mouse parvovirus (MPV), and *Pasteurella pneumotropica* [4, 7, 33, 34, 36, 42]. The embryos were washed using different washing procedures and transferred to pseudopregnant recipients. Pathogens or their antibodies were detected neither in the foster mothers nor in the offspring. The International Embryo Transfer Society (IETS) recommends a minimum of 10 washing steps with at least 100-fold dilution of the previous wash, resulting in a dilution of at least $1 \times 10^{20}$ [35]. In the present study, likewise, 10 steps with at least a 100-fold dilution were included in the IVF-ET procedure.

In this study, MHV-A59 with a virus titer of $10^8$ $TCID_{50}$ per ml was used for the experiments. At first, as a positive control, IVF with the continuous presence of MHV in HTF, KSOM, and M2 media was performed. Seroconversion was detected in 10 of 14 recipient mice and 100% of pups from positive tested for MHV recipients showed maternally derived antibodies to MHV. In contrast, results of experimental group 2 showed that, when $10^8$ $TCID_{50}$ of MHV-A59 per ml were added only to the fertilization medium, the virus was diluted after 10 washing steps to a concentration that was too low to cause infection and seroconversion in the recipients or offspring. PCR examination of the remaining washing media revealed the absence of virus after five washing steps (Fig. 2). A crucial point of each washing step in the present study was the use of a low number of oocytes washed per medium change, which allowed an effective washing regime. This number was used in accordance with the IVF protocol described by Sztein et al. [17], who incubated oocytes from 3–5 females per fertilization dish.

An intact zona pellucida serves as an effective barrier against infection of embryos [35]. Carthew et al. [34] showed that zona pellucida-free mouse embryos exposed to MHV had infected trophoblast cells, but zona-intact embryos were resistant to infection. In our study, we used laser-microdissected oocytes to determine the protecting role of a partially disrupted zona pellucida against MHV infection. After five washing steps, MHV-A59 was no longer detected by PCR. Furthermore, antibodies to MHV were not detected in embryo recipients nor in the offspring. These results indicate that there was no risk of MHV-A59 transmission via our IVF-ET procedure for morphologically intact as well as for partially disrupted oocytes.

It is commonly known that certain viruses, such as bovine herpesvirus BHV-1 and pseudorabies virus, can adhere firmly to the zona pellucida so that even 10 washing steps may fail to remove them [35]. In such cases, washing procedure and brief trypsin treatment have been shown to remove most of the problematic viruses [43]. Likewise, incubation of embryos in medium containing antibiotics for at least 24 h will normally be effective against bacteria and some mycoplasmas [44]. Therefore, on the basis of our results, the possibility of transmission of other common murine viruses, such as mouse parvovirus (MPV) or mouse minute virus (MMV), during IVF-ET procedure cannot be excluded. Just as well, the risk of transmission of other respiratory or enterotropic MHV strains during this procedure remains unresolved and should be investigated.

Furthermore, we examined in our study the consequences of the presence of MHV-A59 in IVF media for the cleavage rate and the percentage of live offspring. The cleavage rate for the control group was 51.1%, which corresponds to the cleavage rate for frozen sperm of the C3HeB/FeJ strain. Marschall et al. [9] found for this strain a cleavage rate of 49.5%. After the continuous presence of MHV throughout the IVF-ET procedure, the cleavage rate was 43.0% ($P > 0.05$ in comparison with the control group). When MHV-A59 was present only in the fertilization medium (experimental group 2), the cleavage rate was 39.9% and significantly lower ($P < 0.001$) than that in the control group. Vanroose et al. [45] used a bovine IVF-ET system to examine the effect of bovine herpesvirus-1 (BHV-1) and bovine viral diarrhea virus (BVDV) on IVF and blastocyst formation. They found that the cleavage rate was significantly lower if virus was present in each step of the IVF-ET procedure or only during fertilization. The tendency toward a reduction of the cleavage rate as observed in our study indicates, therefore, that the infectious virus particles might affect the sensitive fertilization process or that a nonviral component of the virus stock might cause the observed effect, as previously reported by Neighbor and Fraser [46].

Laser-microdissected oocytes fertilized in the absence of MHV-A59 led to a dramatic increase of the cleavage rates (73.0%) compared with laser-microdissected oocytes fertilized in the presence of MHV (39.9%; $P < 0.001$). This indicates that the virus particles might have a higher impact on the fertilization rate of laser-microdissected than intact oocytes. However, both groups had a comparatively low birth rate (3.3% and 6.8%, respectively), which is possibly due to impairment of embryonic development by laser microdissection. In contrast with this, there were no significant differences in the percentage of live offspring between the control group and experimental groups 1 and 2 (16.1%, 13.8%, and 15.0%, respectively). On the other hand, the main focus of this study was to determine the effect of IVF with MHV on seroconversion of embryo recipients and their progeny. Therefore, additional examinations would be necessary to confirm the effect of MHV on the cleavage rate and embryo development.

Taken together, the results showed that 10 washing steps in our IVF-ET procedure prevented MHV infection of recipients and their progeny when MHV-positive fertilization medium was used, even when the zona pellucida was partially disrupted by laser microdissection. Addition of virus to the IVF medium might affect, on a small scale, fertilization rates but has no impact on embryonic development. Finally, it can be

concluded that, by strict use of a tested standard washing procedure in the IVF-ET system, transmission of MHV-A59 can be avoided.

## ACKNOWLEDGMENTS

We thank L. Jennen and H. Wehnes for the scanning electron microscopy; K. Reindl for excellent technical assistance; J. Needham (TML North Harrow, U.K.) for collaboration and support; V. Blanquet for help with implementing the laser microdissection technology; H. Fuchs for statistical analysis; and T. Lisse, J. Sztein, and J. Favor for valuable comments on this manuscript.

## REFERENCES

1. Kraft V, Deeny AA, Blanchet HM. Recommendations for health monitoring of mouse, rat, hamster, guinea pig and rabbit breeding colonies. Lab Anim 1994; 28:1–12.
2. Nicklas W, Baneux P, Boot R, Decelle T, Deeny AA, Fumanelli M, Illgen-Wilcke B. Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab Anim 2002; 36: 20–42.
3. Weir EC, Bhatt PN, Barthold SW, Cameron GA, Simack PA. Elimination of mouse hepatitis virus from a breeding colony by temporary cessation of breeding. Lab Anim Sci 1987; 37:455–458.
4. Reetz IC, Wullenweber-Schmidt M, Kraft V, Hedrich HJ. Rederivation of inbred strains of mice by means of embryo transfer. Lab Anim Sci 1988; 38:696–701.
5. Schenkel J. Transgene Tiere. Heidelberg, Berlin, Oxford: Spektrum Akademischer Verlag; 1995.
6. Morrell JM. Techniques of embryo transfer and facility decontamination used to improve the health and welfare of transgenic mice. Lab Anim 1999; 33:201–206.
7. Van Keuren ML, Saunders TL. Rederivation of transgenic and gene-targeted mice by embryo transfer. Transgenic Res 2004; 13:363–371.
8. Mobraaten LE. Mouse embryo cryobanking. J In Vitro Fert Embryo Transf 1986; 3:28–32.
9. Marschall S, Huffstadt U, Balling R, Hrabe de Angelis M. Reliable recovery of inbred mouse lines using cryopreserved spermatozoa. Mamm Genome 1999; 10:773–776.
10. Thornton CE, Brown SD, Glenister PH. Large numbers of mice established by in vitro fertilization with cryopreserved spermatozoa: implications and applications for genetic resource banks, mutagenesis screens, and mouse backcrosses. Mamm Genome 1999; 10:987–992.
11. Hrabe de Angelis M, Balling R. Large scale ENU screens in the mouse: genetics meets genomics. Mutat Res 1998; 400:25–32.
12. Nolan PM, Peters J, Vizor L, Strivens M, Washbourne R, Hough T, Wells C, Glenister P, Thornton C, Martin J, Fisher E, Rogers D, Hagan J, Reavill C, Gray I, Wood J, Spurr N, Browne M, Rastan S, Hunter J, Brown SD. Implementation of a large-scale ENU mutagenesis program: towards increasing the mouse mutant resource. Mamm Genome 2000; 11:500–506.
13. Hrabe de Angelis MH, Flaswinkel H, Fuchs H, Rathkolb B, Soewarto D, Marschall S, Heffner S, Pargent W, Wuensch K, Jung M, Reis A, Richter T, Alessandrini F, Jakob T, Fuchs E, Kolb H, Kremmer E, Schaeble K, Rollinski B, Roscher A, Peters C, Meitinger T, Strom T, Steckler T, Holsboer F, Klopstock T, Gekeler F, Schindewolf C, Jung T, Avraham K, Behrendt H, Ring J, Zimmer A, Schughart K, Pfeffer K, Wolf E, Balling R. Genome-wide, large-scale production of mutant mice by ENU mutagenesis. Nat Genet 2000; 25:444–447.
14. Marschall S, Hrabe de Angelis M. Cryopreservation of mouse spermatozoa: double your mouse space. Trends Genet 1999; 15:128–131.
15. Nakagata N. Cryopreservation of mouse spermatozoa. Mamm Genome 2000; 11:572–576.
16. Wakayama T, Tanemura K, Suto J, Imamura K, Fukuta K, Mori H, Kuramoto K, Kurohmaru M, Hayashi Y. Production of term offspring by in vitro fertilization using old mouse spermatozoa. J Vet Med Sci 1995; 57:545–547.
17. Sztein JM, Farley JS, Mobraaten LE. In vitro fertilization with cryopreserved inbred mouse sperm. Biol Reprod 2000; 63:1774–1780.
18. Nagy A, Gertsenstein M, Vintersten K, Behringer R. Manipulating the mouse embryo. New York: Cold Spring Harbor Laboratory Press; 2003.
19. Marschall S, Hrabe de Angelis M. In vitro fertilization/cryopreservation. Methods Mol Biol 2003; 209:35–50.
20. Nakagata N, Takeshima T. Cryopreservation of mouse spermatozoa from inbred and F1 hybrid strains. Jikken Dobutsu 1993; 42:317–320.
21. Depypere HT, McLaughlin KJ, Seamark RF, Warnes GM, Matthews CD. Comparison of zona cutting and zona drilling as techniques for assisted fertilization in the mouse. J Reprod Fertil 1988; 84:205–211.
22. Payne D. Micro-assisted fertilization. Reprod Fertil Dev 1995; 7:831–839.
23. Nakagata N, Okamoto M, Ueda O, Suzuki H. Positive effect of partial zona-pellucida dissection on the in vitro fertilizing capacity of cryopreserved C57BL/6J transgenic mouse spermatozoa of low motility. Biol Reprod 1997; 57:1050–1055.
24. Kawase Y, Iwata T, Ueda O, Kamada N, Tachibe T, Aoki Y, Jishage K, Suzuki H. Effect of partial incision of the zona pellucida by piezo-micromanipulator for in vitro fertilization using frozen-thawed mouse spermatozoa on the developmental rate of embryos transferred at the 2-cell stage. Biol Reprod 2002; 66:381–385.
25. Kawase Y, Aoki Y, Kamada N, Jishage K, Suzuki H. Comparison of fertility between intracytoplasmic sperm injection and in vitro fertilization with a partial zona pellucida incision by using a piezo-micromanipulator in cryopreserved inbred mouse spermatozoa. Contemp Top Lab Anim Sci 2004; 43:21–25.
26. Enginsu ME, Schutze K, Bellanca S, Pensis M, Campo R, Bassil S, Donnez J, Gordts S. Micromanipulation of mouse gametes with laser microbeam and optical tweezers. Hum Reprod 1995; 10:1761–1764.
27. Yanagimachi R. Intracytoplasmic sperm injection experiments using the mouse as a model. Hum Reprod 1998; 13(suppl 1):87–98.
28. Szczygiel MA, Kusakabe H, Yanagimachi R, Whittingham DG. Intracytoplasmic sperm injection is more efficient than in vitro fertilization for generating mouse embryos from cryopreserved spermatozoa. Biol Reprod 2002; 67:1278–1284.
29. Germond M, Nocera D, Senn A, Rink K, Delacretaz G, Fakan S. Microdissection of mouse and human zona pellucida using a 1.48-microns diode laser beam: efficacy and safety of the procedure. Fertil Steril 1995; 64:604–611.
30. Germond M, Nocera D, Senn A, Rink K, Delacretaz G, Pedrazzini T, Hornung JP. Improved fertilization and implantation rates after non-touch zona pellucida microdrilling of mouse oocytes with a 1.48 microm diode laser beam. Hum Reprod 1996; 11:1043–1048.
31. Liow SL, Bongso A, Ng SC. Fertilization, embryonic development and implantation of mouse oocytes with one or two laser-drilled holes in the zona, and inseminated at different sperm concentrations. Hum Reprod 1996; 11:1273–1280.
32. Hollis A, Rastegar S, Descloux L, Delacretaz G, Rink K. Zona pellucida microdrilling with a 1.48 microns diode laser. IEEE Eng Med Biol Mag 1997; 16:43–47.
33. Carthew P, Wood MJ, Kirby C. Elimination of Sendai (parainfluenza type 1) virus infection from mice by embryo transfer. J Reprod Fertil 1983; 69: 253–257.
34. Carthew P, Wood MJ, Kirby C. Pathogenicity of mouse hepatitis virus for preimplantation mouse embryos. J Reprod Fertil 1985; 73:207–213.
35. Stringfellow DA, Seidel SM. Manual of The International Embryo Transfer Society. Savoy, IL: International Embryo Transfer Society (IETS); 1998.
36. Suzuki H, Yorozu K, Watanabe T, Nakura M, Adachi J. Rederivation of mice by means of in vitro fertilization and embryo transfer. Exp Anim 1996; 45:33–38.
37. Spearman C. The method of the 'right and wrong cases' ('constant stimuli') without Gauss's formulae. Br J Psychol 1908; 2:227–242.
38. Kaerber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch Exp Pathol Pharmakol 1931; 162:480–483.
39. Mahabir E, Jacobsen K, Brielmeier M, Peters D, Needham J, Schmidt J. Mouse antibody production test: can we do without it? J Virol Methods 2004; 120:239–245.
40. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 1985; 44:493–498.
41. Taylor K, Copley CG. Detection of rodent RNA viruses by polymerase chain reaction. Lab Anim 1994; 28:31–34.
42. Scavizzi F, Raspa M. Tissue distribution and duration of mouse hepatitis virus in naturally infected immunocompetent ICR (CD-1) and immunodeficient athymic nude-nu mouse strains used for ovarian transplantation and in vitro fertilization. Lab Anim 2004; 38:189–199.
43. Trachte E, Stringfellow D, Riddell K, Galik P, Riddell M Jr, Wright J.

Washing and trypsin treatment of in vitro derived bovine embryos exposed to bovine viral diarrhea virus. Theriogenology 1998; 50:717–726.
44. Hill AC, Stalley GP. Mycoplasma pulmonis infection with regard to embryo freezing and hysterectomy derivation. Lab Anim Sci 1991; 41: 563–566.
45. Vanroose G, Nauwynck H, Van Soom A, Vanopdenbosch E, De Kruif A. Effect of bovine herpesvirus-1 or bovine viral diarrhea virus on development of in vitro-produced bovine embryos. Mol Reprod Dev 1999; 54:255–263.
46. Neighbour PA, Fraser LR. Murine cytomegalovirus and fertility: potential sexual transmission and the effect of this virus on fertilization in vitro. Fertil Steril 1978; 30:216–222.