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Production of Virus-Free Seronegative Pups from Murine Embryos Arising from In Vitro Fertilization with Mouse Minute Virus-Exposed Spermatozoa

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ABSTRACT

In the present study, the risk of transmission of mouse minute virus (MMV) to recipients of murine embryos arising from in vitro fertilization (IVF) of oocytes with MMV-exposed spermatozoa and to resulting pups was evaluated. Also, the time of seroconversion of recipients and pups was investigated. To achieve this goal, IVF of oocytes with cryopreserved spermatozoa from the inbred C3HeB/FeJ mouse strain was performed, and the resulting embryos were transferred to suitable Swiss recipients. Three groups were investigated: 1) oocytes or the developing embryos were continuously exposed to 10^4 $TCID_{50}$ MMVp per milliliter in the fertilization (human tubal fluid [HTF]), culture (KSOM), and embryo transfer (M2) media (positive control); 2) oocytes and spermatozoa were exposed to MMVp in the HTF medium only and transferred after a standard washing procedure with 10 washing steps in virus-free KSOM and M2; and 3) oocytes and spermatozoa were exposed to virus-free HTF, KSOM, and M2 (negative control). To detect antibodies to MMV in recipients and progeny, serological analyses were performed by ELISA on Days 14, 21, 28, and 42, and on Days 42 and 63, respectively, after embryo transfer. The presence of MMV in the washing drops was analyzed by PCR and an in vitro infectivity assay, while organs of some recipients and pups were analyzed by PCR. Using 10^4 of the tissue culture infective dose of MMVp per millilitre in the fertilization medium only, the present results demonstrate that 10 washing steps in the IVF-ET procedure are sufficient to remove the virus to a noninfectious dose, producing MMV-free seronegative recipients and pups. As such, there is minimal risk of transmission of MMV to recipients and pups if spermatozoa become contaminated with such viral loads.

assisted reproductive technology, embryo transfer, health monitoring, in vitro fertilization, mouse minute virus, mouse, uterus

INTRODUCTION

In mouse husbandry and breeding programs, it is essential to preserve the germ lines. Depending on the strain of mice, this is usually achieved by cryopreservation of spermatozoa [1] and/or embryos [2, 3]. Where cryopreservation of spermatozoa is performed, the mouse line or strain is then rederived by performing in vitro fertilization and embryo transfer (IVF-ET) [4, 5]. Recipients are health monitored and, provided that they are negative for relevant microorganisms, their progeny are released into full-barrier holding areas.

An intact zona pellucida (ZP) is regarded as a mechanism for preventing pathogen transmission during embryo transfer [4, 6–12]. Furthermore, the International Embryo Transfer Society recommends that embryos be washed 10 times prior to transfer to recipients [13]. No transmission of mouse hepatitis virus was found when in vitro- and in vivo-produced mouse embryos were transferred to recipients [4, 7, 8, 14, 15]. However, in vivo-produced embryos which had been exposed in vitro to a minimum of 10^4 of the tissue culture infective dose ($TCID_{50}$) of MMVp per milliliter and washed 10 times led to seropositive recipients [15].

Mouse minute virus (MMV) is a nonenveloped, linear, positive-strand DNA virus of the Parvoviridae family having a diameter of approximately 20 nm [16]. Breeding mice become infected during the second and third months of life [17], and the outcome of natural infection in immunocompetent mice is usually asymptomatic. During acute infection, virus replication occurs in the small intestine, lymphatic organs, and the liver. The virus can persist in the mesenteric lymph nodes for a long period and is transmitted primarily by urinary and/or fecal excretion [17]. MMV is highly resistant to environmental factors and even to a number of disinfectants [18, 19].

For cryopreservation of sperm, the caudae epididymides and vasa deferentia, together with their blood vessels, are removed from the mouse and torn to allow release of the spermatozoa. Thus, if males are infected with MMV, the possibility exists that this virus will also be present in the sperm suspension. In the fertilization dish, MMV may become attached directly to the cumulus cells and/or the oocytes. Furthermore, because spermatozoa penetrate the ZP to enable fertilization, they may also serve as vectors for transmission of MMV into the oocyte. The presence of MMV on or within the in vitro-produced embryos even after washing may lead to MMV-positive recipients and pups.

The aim of the present study was to determine whether the presence of MMVp in the IVF procedure leads to attachment of this virus to the resulting embryos and, consequently, to infection and seroconversion of recipients and pups. The relevance of IVF-ET as a safe means to eliminate MMV from infected colonies and the subsequent health monitoring of recipients after embryo transfer are discussed, because concerted efforts are made to prevent pathogen transmission and to maintain SPF barrier areas.

MATERIALS AND METHODS

Cells and Viruses

The MMVp virus stock (VR-1346), its producer cell line A9 (CCL-1.4), and L929 cells (CCL-1) were obtained from the American Type Culture Collection (Manassas, VA). A9 cells were used for propagation, and L929 cells were used for titration. A9 and L929 cell cultures were maintained in Dulbecco

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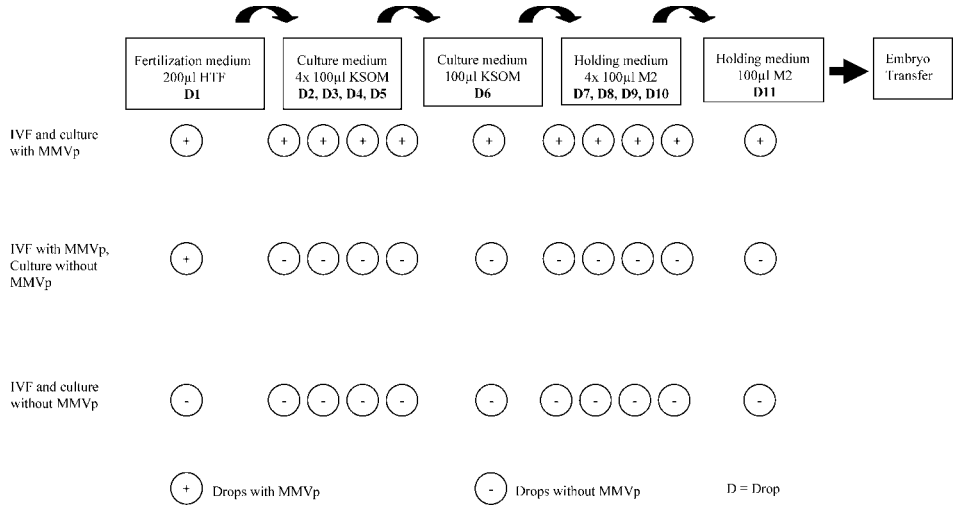

FIG. 1. The experimental design for in vitro fertilization and culture in the present study. Oocytes were fertilized in HTF, washed four times in KSOM, cultured overnight in KSOM, washed four times in M2, and kept in M2 prior to embryo transfer to suitable recipients. Oocytes or the developing embryos were continuously exposed to 10^4 $TCID_{50}$ MMVp per milliliter in HTF, KSOM, and M2, or oocytes and spermatozoa were exposed to MMVp in HTF only and transferred after a standard washing procedure with 10 washing steps in virus-free KSOM and M2. Oocytes and spermatozoa were also exposed to virus-free HTF, KSOM, and M2.

modified Eagle medium (DMEM) supplemented with 4.5 g/L D-glucose/L-glutamine and 10% (A9) or 5% (L929) heat-inactivated fetal calf serum (FCS). Propagation of virus stocks was performed in 75-cm^2 cell culture flasks (Nunc, Roskilde, Denmark) at 37°C using 5% CO_2 in a humidified atmosphere. Cultures of permissive cells were infected with the appropriate virus for 1 h, followed by removal of the virus suspension and replacement with 10 ml cell culture medium. After 5 days' culture, MMVp-infected permissive cells were frozen in their culture flasks. They were subjected to three freeze-thaw cycles to allow release of virus. The contents of the flasks were centrifuged at $3000 \times g$ for 5 min to separate virus from cell debris. The supernatant was passed through a Minisart filter having a pore size of 0.20 µm (Sartorius, Göttingen, Germany). For titration, L929 cells were seeded in 96-well plates at a concentration of 3×10^3 per well and cultured overnight. After removal of the culture medium, 12-fold wells were infected with 100 µl of each of the 10-fold dilutions up to 10^{-10} of the virus suspension. The cytopathic effect, observed as detachment of cells, was determined on the sixth day of culture. The mean $TCID_{50}$ for the viral stock was calculated according to the Spearman-Kaerber method [20, 21]. The MMVp stocks used in this study had titers of 10^6 $TCID_{50}$ per milliliter and were stored at −80°C until used.

Mice and Husbandry

Outbred Crl:CD1(Icr) and inbred C3HeB/FeJ mice were bred in a full barrier unit at the GSF animal facilities. Breeding colonies were kept in filter-topped Type II Makrolon cages at a temperature of 20–24°C, a humidity of 50%–60%, 20 air exchanges per hour, and a 12L:12D cycle. Wood shavings (Altromin, Lage, Germany) were provided as bedding. Mice were fed a standardized mouse diet (1314; Altromin) and provided drinking water ad libitum.

Before entering a mouse room, staff were clothed in a clean suit and wore disposable gloves, bonnets, and face masks. During routine weekly changes of cages including lids, wire bars, and water bottles in class II laminar flow changing stations, mice were transferred to new cages using forceps padded with silicone tubing. Forceps were disinfected after each cage change with 70% ethanol. All materials, including the Makrolon cages, lids, feeders, bottles, bedding, and water, were autoclaved before use.

Microbiological examination was performed every 6 wk using male Crl:CD1(Icr) sentinels from the colony as described [22]. Briefly, aliquots of approximately 5 cm^3 of soiled bedding were taken from each used cage on a rack. These aliquots were mixed in a sterile box with an equivalent amount of new sterile bedding, and the resultant mixture was distributed to the sentinel cage of the same rack over a period of 12 wk. The serological examinations were performed according to the annual standard recommended by FELASA (Federation of Laboratory Animal Science Associations) [23] with the addition of Leptospira serogroups, ballum, canicola, hebdomadis and icterohaemorrhagiae, K virus, lactate dehydrogenase virus, polyoma virus, mouse thymic virus, hantaviruses [24], and murine norovirus. The mice were found consistently negative for all of the above-mentioned infectious agents, including MMV.

Experimental and control mice were kept in autoclaved, individually ventilated cages (IVCs; VentiRacks; BioZone, Margate, U.K.) under positive pressure and the conditions stated above. All animal manipulations were performed in a class II laminar flow biological safety cabinet (Heraeus Instruments GmbH, Munich, Germany). All animal studies were approved by the GSF Institutional Animal Care and Use Committee and the Government of Upper Bavaria, Germany (211-2531–8/02).

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 containing 3% skim milk and 18% raffinose. The cryoprotectant solution was prepared as described [25]. The tissues were torn with sterile injection needles (Braun, Melsungen, Germany) in a culture dish and the spermatozoa were allowed to disperse for 5 min at 37°C in a moisture-saturated atmosphere of 5% CO_2/95% air in an incubator. The dish was gently shaken until a homogeneous sperm suspension was obtained. The sperm was loaded in 17.5-µl aliquots in 11–12 straws (0.25 ml; Minitüb, Tiefenbach, Germany). Straws were heat sealed and placed in liquid nitrogen vapor at −120°C. Ten minutes later, the straws were stored in liquid nitrogen. For thawing, one straw each from two different males were put in a water bath at 37°C for 2 min, and the contents were expelled and mixed in a culture dish. A volume of 3 µl of thawed spermatozoa was placed in 197-µl drops of pre-equilibrated virus-free or virus-containing human tubal fluid (HTF) [26] medium under embryo-tested mineral oil (Sigma-Aldrich, Taufkirchen, Germany). The 200-µl HTF drops with thawed spermatozoa were incubated for 30 min at 37°C in a moisture-saturated atmosphere of 5% CO_2/95% air in an incubator and used for IVF (fertilization drops). The motility and progression of the sperm were determined using a Hamilton Thorne IVOS computerized semen analyzer (Hamilton Thorne, Beverly, MA) as described [5]. The concentration of the spermatozoa in the HTF drops ranged from 10 to 60×10^6/ml. Spermatozoa for experiments with MMVp in the fertilization medium only originated from different males, as did the spermatozoa for experiments where MMVp was present both in the fertilization and culture media.

In Vitro Fertilization and Washing Procedure

Inbred C3HeB/FeJ female mice 21–28 days old were superovulated by intraperitoneal injection of 5 IU of eCG (Intergonan 1000; Intervet, Unterschleißheim, Germany) at 1800 h followed by 5 IU of hCG (Ovogest 1500; Intervet) 48 h later. Mice were killed by cervical dislocation 13–14 h after the hCG injection, and the oviducts were removed. The cumulus-oocyte complexes were isolated from the oviducts in a 500-µl HTF drop under mineral oil and added to the fertilization dishes. Complexes from five females were co-incubated with spermatozoa in a fertilization drop. The experimental design for IVF and culture is summarized in Figure 1. Three groups were investigated: 1) oocytes or the developing embryos were continuously exposed to 10^4 $TCID_{50}$ MMVp per milliliter in the fertilization (HTF), culture (KSOM, Powdered Media Kit; Metachem Diagnostics Ltd., Northampton, UK), and embryo transfer (M2, Sigma, Deisenhofen, Germany) media (positive control); 2) oocytes and spermatozoa were exposed to MMVp in the HTF medium only and transferred after a standard washing procedure with 10 washing steps in virus-free KSOM and M2; and 3) oocytes and spermatozoa were exposed to virus-free HTF, KSOM, and M2 (negative control). Experimental and control embryos were cultured in separate dishes.

After 5 h co-incubation of gametes at 37°C in a moisture-saturated atmosphere of 5% CO_2/95% air in an incubator, the oocytes from each fertilization drop were washed through each of four 100-µl drops of KSOM and

TABLE 1. Reproductive data after transfer of embryos from IVF with gametes exposed to MMVp.

Experimental group	No. of oocytes coincubated	No. of two-cells (%)	No. of two-cells transferred	No. of recipients littering/no. of recipients (%)	No. of live young (%)[a]
In vitro fertilization and culture with MMVp	419	229 (55)	200	2/10 (20)	4 (2)
In vitro fertilization without MMVp	373	154 (41)	120	4/6 (67)	9 (7.5)
In vitro fertilization with MMVp (serology)	902	327 (36)	280	6/14 (43)	12 (4.3)
In vitro fertilization without MMVp (serology)	942	238 (25)	200	4/10 (40)	9 (4.5)
In vitro fertilization with MMVp (serology and PCR)	2026	563 (28)	460	9/23 (39)	22 (5)
In vitro fertilization without MMVp (serology and PCR)	1093	278 (25)	180	5/9 (56)	20 (11)

[a] Number of live mice from the total number of embryos transferred.

were cultured overnight in the fifth 100-μl drop of KSOM under mineral oil. Twenty-four hours after commencement of the IVF procedure, the number of morphologically intact 2-cell embryos having blastomeres of approximately the same size, the number of 1-cell embryos, and dead and fragmented oocytes were counted. The cleavage rate was calculated as the percentage of 2-cell embryos obtained from the total number of intact oocytes placed in a fertilization dish. The 2-cell embryos from each fertilization drop were washed through each of four 100-μl drops of M2 medium, and 80–100 cells from different fertilization dishes were placed in the fifth 100-μl drop of M2. In the IVF procedure, the oocytes and embryos were washed 10 times through the corresponding media. Embryos were transferred to Day 0.5 pseudopregnant recipients as described [27]. Thus, from fertilization to embryo transfer, a total of 11 drops was obtained, and this is referred to as a set of drops in this report (Fig. 1).

A new sterile micropipette was used between each IVF dish and for each recipient. The dilution factor between washing drops was approximately 1:100. The maximum number of oocytes washed with the same pipette was 100. In the group where IVF and culture were performed with MMVp, experiments were carried out on 2 different days. Where IVF alone was performed with MMVp, experiments were performed on 15 different days.

Embryo Transfer

A total of 10 embryos from each experimental group was transferred by an experienced experimenter into each oviduct of Day 0.5 pseudopregnant Crl:CD1(Icr) recipients as described [27]. Negative control embryos were transferred before experimental embryos. The average volume of medium including embryos transferred was 0.2 μl per recipient. Surgery was performed under general anesthesia with appropriate analgesic support as described [27]. After embryo transfer, the recipients were housed singly in IVCs. Pups were weaned at 3 wk of age and housed singly in IVCs.

Serological Analysis

To determine if the recipients seroconverted, blood was collected from the tail vein on Days 14, 21, 28, and 42 after embryo transfer. Sera from progeny were prepared on Days 42 and 63 after embryo transfer and analyzed. Sera were not inactivated prior to the test. Samples were diluted 1:60 in the serum diluent that was composed of 99 ml PBS (Oxoid, Hants, UK) containing 5 g nonfat dry bovine milk, 5 mg Gentamycin sulphate (Sigma, Dorset, UK), and 33 μl Antifoam A concentrate (Sigma). Sera were tested for specific antibodies (IgG whole molecule) to MMV by use of an ELISA using control non-viral-coated and viral-coated plates and negative and positive serum (Charles River Laboratories, Wilmington, MA). The optical density (OD) was read at 492 nm with a Multiskan ELISA plate reader (Thermo Life Sciences, Hampshire, UK). Serological results for MMV were positive when the OD was greater than 0.399.

Virological Examination of Washing Drops and Embryos

The used media were stored in sterile tubes at −80°C until analyzed. In total, drops from 10 sets of IVF and culture-positive dishes with negative control drops from 6 sets of dishes, and drops from 45 sets of IVF-positive dishes with drops from 25 sets of negative control dishes were analyzed. All drops were diluted 1:10 with DMEM and 10% FCS and tested for the presence of MMVp by PCR, and for virus infectivity by in vitro infectivity assay in cell culture using the protocols described [15, 28]. DMEM containing 10% FCS was used as the diluent to facilitate cell culture. The in vitro infectivity assay with L929 cells is able to detect 1 $TCID_{50}$ of MMV (data not shown).

Briefly, for the in vitro infectivity assay, L929 cells were seeded in 96-well plates at a concentration of 3×10^3 per well and cultured overnight. After removal of the culture medium, 2-fold wells were infected with 100 μl of each dilution. The cytopathic effect, observed as detachment of cells resulting from the MMVp infection, was determined on the sixth day of culture.

For PCR, 200 μl of the diluted drops were analyzed. Total DNA from each diluted drop was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The primers used were those designed by Bootz et al. [29] as follows: 5′-GAGCGCCATCTAGTGAGC-3′ (forward) and 5′-ATTTGCCTGTGCTGGCTG-3′ (reverse), yielding a 483-bp product. A double-distilled water sample served as a negative PCR control. PCR was performed in a total volume of 20 μl using Taq DNA polymerase (Qiagen) for 40 cycles in a thermo cycler (Biometra; Biomedizinische Analytik GmbH, Göttingen, Germany). Denaturation was performed at 94°C for 4 min. Each cycle consisted of 94°C (30 sec), 55°C (30 sec), and 72°C (30 sec). The last cycle was followed by a 7-min extension period at 72°C. PCR products (10 μl) were mixed with 2 μl loading buffer (MBI Fermentas, St. Leon-Rot, Germany), electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The PCR assay is 10^6 times more sensitive than the in vitro infectivity assay in cell culture and has a limit of detection of 10^{-6} $TCID_{50}$.

In addition, in the group where IVF alone was performed in the presence of MMVp, 1, 2, 3, 14, 19, 20, 20, and 20 embryos were analyzed for the presence of MMVp using PCR and an in vitro infectivity assay. In the negative control group, 6, 7, 20, 20, and 20 embryos were analyzed. Prior to DNA extraction and PCR as described above for the media, embryos were frozen and thawed three times to allow release of the virus.

PCR Analysis of Organs from Recipients and Pups

To determine if recipients and pups were MMV-negative, in addition to serology, the mesenteric lymph nodes and small intestines from some of the recipients and pups were examined for the presence of MMV by PCR, as described above. Mice were taken from the group where only IVF was performed in the presence of MMVp and from the negative control group. Organs were collected on Day 42 from recipients that littered and from their pups on Day 42 or 63 after embryo transfer. DNA was extracted using the QIAamp DNA mini kit according to the manufacturer's instructions.

RESULTS

Reproductive Data

IVF and culture or IVF alone were performed in the presence of MMV, and the resulting embryos were transferred to suitable recipients. The reproductive data from the IVF and embryo transfers are presented in Table 1. With respect to IVF and culture in the presence of MMVp, the cleavage rate, defined as the percentage of 2-cells developing, was 55%. From 10 recipients, 2 littered a total of 4 live pups. The cleavage rate of the negative control group was 41%. From 6 recipients, 4 littered a total of 9 live pups. With respect to IVF in the presence of MMVp where serology only was performed, the cleavage rate was 36%. From 14 recipients, 6 littered a total of 12 live pups. The cleavage rate of the negative control group was 25%. From 10 recipients, 4 littered a total of 9 live pups. Where IVF was done in the presence of MMVp and both serology and PCR were performed, the cleavage rate was 28%. From 23 recipients, 9 littered a total of 22 live pups. The cleavage rate of the negative control group was 25%. From 9 recipients, 5 littered a total of 20 live pups.

TABLE 2. Results of transfer of two-cell embryos into Day 0.5 pseudopregnant recipients following exposure of gametes to 10^4 $TCID_{50}$/ml MMVp during fertilization and seroconversion of recipients and progeny.

	Seropositive mice days after embryo transfer					
	Recipients				Progeny	
Experimental Group	Day 14	Day 21	Day 28	Day 42	Day 42	Day 63
In vitro fertilization and culture with MMVp	3/10	5/10	6/10	6/10	0/4	0/4
In vitro fertilization and culture without MMVp	0/6	0/6	0/6	0/6	0/7	0/7
In vitro fertilization with MMVp (serology)	0/14	0/14	0/14	0/14	0/12	0/12
In vitro fertilization without MMVp (serology)	0/10	0/10	0/10	0/10	0/9	0/9
In vitro fertilization with MMVp (serology and PCR)	0/23	0/23	0/23	0/23	0/22[a]	0/10
In vitro fertilization without MMVp (serology and PCR)	0/9	0/9	0/9	0/9	0/20[b]	0/9

[a] Twelve pups killed for PCR analysis.
[b] Eleven pups killed for PCR analysis.

Serological Data

In the group where IVF and culture were performed in the presence of MMVp, three seropositive recipients were detected by Day 14 after embryo transfer (Table 2). The number increased to five by Day 21 and six by Days 28 and 42. All four pups, which originated from seronegative recipients from this group, were seronegative for MMVp at Days 42 and 63 after embryo transfer. The negative control recipients and their pups did not have any detectable antibodies to MMVp.

In the group where IVF alone was performed in the presence of MMV and only serology was performed, all 14 recipients and their 12 progeny were seronegative throughout the study. The negative control recipients and their pups did not have any detectable antibodies to MMVp.

Where IVF alone was performed in the presence of MMV and serology and PCR were performed, all 23 recipients and their 22 progeny were seronegative throughout the study. The nine negative control recipients and their 20 pups did not have any detectable antibodies to MMVp.

Virological Examination of Remaining Media, Washing Fluids, and Embryos

In the group where IVF and culture were performed in the presence of MMVp, the two sets of 11 drops were positive for MMVp as shown by PCR (Fig. 2A) and in vitro infectivity assay. A total of 4 and 10 embryos tested positive for MMVp by both PCR and the in vitro infectivity assay. The controls (n = 5 and 10) were negative for MMV (data not shown).

Figure 3 shows the data for detection of MMVp using PCR and for the in vitro infectivity assay in the group where only IVF was performed in the presence of MMVp. Some drops were found positive for MMVp with both methods. In the PCR testing of drops from a total of 45 fertilization dishes from which embryos developed, the HTF medium (D1) and Drop 2 (D2) were positive in 45 cases. In D3, 44 drops were positive for MMVp. In D6, the number of positive drops decreased to 38 (Fig. 2, B and C), whereas 23 drops were positive in D7. The number of MMV-positive drops decreased to 4 in D10. Where embryos were held in M2 prior to transfer, MMVp was detected in 4 from 11 drops analyzed (Fig. 2, B and C). Embryos tested negative in all eight cases analyzed.

The in vitro infectivity assay showed that the HTF medium (D1) was always positive for MMVp. The number of dishes that were positive for MMVp in D2, D3, D4, D5, D6, and D7–D10 was 42, 31, 19, 6, 1, and 0, respectively (Fig. 3). Where embryos were held in M2 prior to transfer, MMVp was detected in none of the 11 drops analyzed. The embryos tested negative for MMVp.

From the experimental groups, negative control drops (Fig. 2D) and embryos were consistently negative for MMVp.

PCR Analysis of Organs from Recipients and Pups

PCR analysis of the mesenteric lymph nodes and the intestines of 23 recipients and 12 pups taken on Day 42 and of the remaining 10 pups taken on Day 63 from the group where

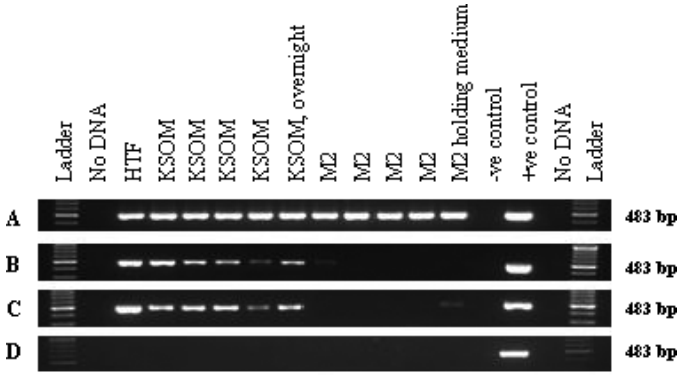

FIG. 2. MMV PCR analysis using the remaining media from the fertilization drop, washing steps, and the holding media from the three experimental groups. **A**) In vitro fertilization and culture with MMVp in all drops. **B**) In vitro fertilization with MMVp in HTF only where M2 was negative. **C**) In vitro fertilization with MMVp in HTF only where M2 was positive. **D**) In vitro fertilization and culture without MMVp. The size of the PCR product is 483 bp.

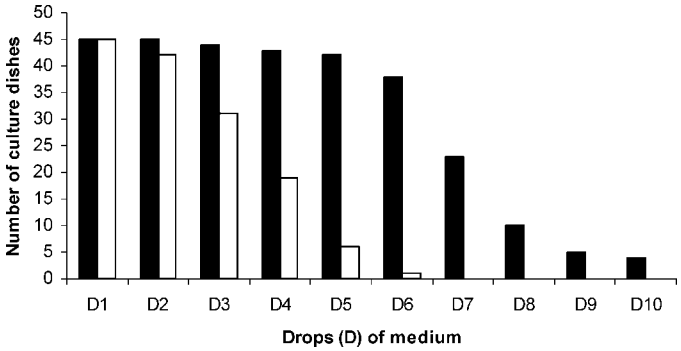

FIG. 3. Number of media drops (D) that were positive for MMVp by PCR (black bars) and by in vitro infectivity assay (white bars) from a total of 45 sets of dishes, where the fertilization dishes contained 10^4 $TCID_{50}$ MMVp per milliliter of HTF (D1). D2 to D5: washing steps with KSOM; D6: overnight culture in KSOM; D7 to D10: washing steps with M2.

IVF was performed in the presence of MMVp did not reveal the presence of MMV. Likewise, 9 control recipients and 11 pups on Day 42 and 9 pups on Day 63 were negative for MMV.

DISCUSSION

In the present study, we investigated the risk of transmission of MMV to Swiss recipients from embryos arising through fertilization of oocytes with MMVp-exposed spermatozoa, and also investigated whether pups were MMV-free. This work was performed under aseptic, routine working conditions for the production of rederived mice that precluded natural exposure to these agents. The results show that recipients of embryos arising from fertilization of oocytes with MMV-exposed spermatozoa did not produce antibodies to this virus and pups were seronegative. Both recipients and pups were MMV-free, as detected by PCR. These data suggest that washing the embryos 10 times, as performed in the present experiment, is sufficient to remove the viral load used in the present study.

The C3HeBFeJ strain of mice was chosen for this study because it is frequently used to produce mutant mice and it can be successfully archived by cryopreservation of spermatozoa and revitalized via in vitro fertilization and embryo transfer. Swiss mice are typically used as embryo transfer recipients at many facilities. In the present study, we used the prototype strain MMVp to extend the results of previous experiments, thereby enabling comparison of data [15, 28].

Previous work [15] showed that in vivo-produced embryos that were co-incubated with 10^4 $TCID_{50}$ MMVp, as used in the present study, and washed 10 times were carriers of the virus. PCR detection of MMV in some washing drops and seroconversion in recipients indicated that washing of embryos through 10 drops sometimes only reduced the viral load 10 to 100-fold. Furthermore, 60 embryos that were previously exposed to 10^6 $TCID_{50}$ MMVp and washed 10 times had a titer of 10^5 $TCID_{50}$ per milliliter after titration on L929 cells [15]. This result is in contradiction to the present data with in vitro-produced embryos. This variation in results may be due to differences in the characteristics of the ZP of in vitro and in vivo-produced embryos. It is known that after fertilization physiological changes in the ZP occur in order to block polyspermy. Micropores having a size of 140-1000 nm in the ZP of murine embryos [30] arise due to removal of the cumulus cells. During the in vitro fertilization process, not all cumulus cells are removed by the time the oocytes are removed from the fertilization drop and washed in preparation for culture. Thus, the number of micropores in the ZP of such oocytes is not expected to be as high as that found in embryos starting from the 2-cell stage, allowing entrapment of a lower number of the virus from the fertilization media. Such a situation may be conducive to adequate removal of the virus by washing to a noninfectious dose for the embryo transfer recipients. In addition, the cumulus cells may have adsorbed some of the virus.

With respect to the experiment where MMVp was present only in the fertilization medium and considering the washing procedure used in the present study, the PCR analysis, which also detects noninfectious virus, showed that there was a reduction in the viral load starting at the second washing step. The viral load continually decreased to Drop 10, where MMV was still found in 4 of the dishes analyzed. The in vitro infectivity assay, which detects infectious virus, showed that there was a steep decrease in the amount of infectious viruses to Drop 6, where only one dish was positive, after which no infectious virus was detected. Although some sequential washing drops were free of MMVp, the holding medium that contained up to 100 embryos for 1 to 2 h was sometimes weak positive by PCR (Fig. 2C), but negative by the in vitro infectivity assay, implicating the presence of noninfectious virus based on the permissive cell line used in the present study or due to higher sensitivity of the PCR. These data showed that embryos sometimes served as vectors for MMVp up to the stage of the holding medium (Fig. 2C). However, analysis of a pool of up to 20 embryos by PCR and the in vitro infectivity assay did not show MMVp. Most likely, MMV from the embryos would have been deposited in the holding medium, and MMV may have been present on the embryos in amounts that are below the limit of detection of the assays used in the present study. The absence of MMV in recipients and pups and the lack of seroconversion confirm the absence of an infective or immunogenic dose by the embryos. Major factors contributing to the reduction of the viral load are certainly the use of new pipettes for each fertilization or culture dish and each washing drop, and the fact that a maximum of approximately 100 oocytes was contained in each dish.

In the group where all media drops contained MMVp, antibodies against MMV were found in 6 of the 10 recipients by Day 28 after embryo transfer without an increase in the number of seropositive recipients at Day 42 after embryo transfer, thus enabling microbiological examination at the time of weaning of the pups. No antibodies against MMV were detected in pups born to seronegative recipients. However, previous work in our laboratory [15] showed that pups receive maternal antibodies from their mothers when the recipients seroconvert by Day 14 after embryo transfer, and this antibody in the pups decreased with time. In the present study, we also confirmed the absence of antibodies in some recipients receiving embryos from virus-containing dishes, implying that although virus is present in the transferred embryos and/or the media, the recipients may not necessarily become infected. As previously reported, such differences may be due to specific and unspecific immune mechanisms of the outbred mice used in this study [28] or antiviral activity in the uterine milieu [31, 32].

For routine in vitro fertilization and embryo transfer work, quality control measures could include PCR analysis of spermatozoa for the presence of viruses prior to their use, biocontainment including the use of micro-isolators, and health monitoring of each recipient.

Our findings show that embryos arising from fertilization of oocytes with MMV-exposed spermatozoa that were washed 10 times led to MMV-free seronegative recipients and pups. As such, the risk of transmission of MMVp by in vitro-produced embryos to recipients during embryo transfer appears to be very low or nonexistent.

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