The association between Foot-and-mouth disease virus and bovine oocytes and embryos during in vitro embryo production.

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List of Abbreviations

BHV-1	= Bovine herpes virus-1
BK-cell culture	= Bovine kidney-cell culture
BVDV	= Bovine virus diarrhoea virus
COC	= Cumulus oocyte complex
CPE	= Cytopathic effect
ELISA	= Enzyme - linked Immunosorbent Assay
FMD	= Foot-and-mouth disease
FMDV	= Foot-and-mouth disease virus
IETS	= International Embryo Transfer Society
IVC	= *In vitro* Culture
IVF	= *In vitro* Fertilisation
IVM	= *In vitro* Maturation
IVP	= *In vitro* Produced
KNP	= Kruger National Park
l	= Litre
LH	= Luteinizing Hormone
M	= Molar
MES	= 2-[N-Morpholino]ethanesulfonic acid
ml	= millilitre
mm	= millimetre
nm	= nanometre
PBS	= Phosphate Buffered Saline
PCR	= Polymerase Chain Reaction
pfu	= plaque forming units

PKCC	= Pig kidney–cell culture
R.S.A	= Republic of South Africa
SOF	= Synthetic Oviduct Fluid
TALP	= Tyrode's Albumin–Lactate-Pyruvate
TCM199-H	= Tissue culture medium 199-H
µl	= microlitre
ZP	= Zona-pellucida

1 INTRODUCTION

1.1 A need to prevent transmission of Foot-and-mouth disease virus with embryo production.

Foot-and-mouth disease virus (FMDV) is a highly contagious viral infection affecting almost exclusively ruminants and pigs (1). It is characterized by high morbidity, low mortality, and by vesicles and erosions in the mucosa of the mouth and skin of the interdigital spaces and the coronary bands (1). It will continue to be the most important disease of livestock in southern Africa because of the potential crippling effect on the economy. Countries free from the disease have control measures in place to prevent importation of the disease. In the case of an outbreak, export of agricultural products is severely affected (1-3).

The operational costs for the National Department of Agriculture to contain and manage the outbreaks in South Africa from September 2000 to February 2001 amounted to ±ZAR 75 million. The total operational costs are estimated at ZAR 90 million. The indirect losses because of the outbreak (export bans etc.) amounts to much more (3).

This work can serve as a model to produce FMDV free *in vitro* produced (IVP) embryos. Foot-and-mouth disease (FMD) is endemic to the Kruger National Park (KNP) in the Republic of South Africa (R.S.A) where the virus persists in the Cape or African buffalo population. Once an animal is infected, the infection is regarded as lifelong, because a carrier state exists in some animals (1,3-8). The rest of the R.S.A (R.S.A other than the KNP and control areas around it) is free from FMD (8). A buffer zone and control areas exist around the KNP and along the fence on the Northern border of the R.S.A to monitor a possible outbreak and spread of FMD. Figure 1.1 shows the FMD control areas of the R.S.A. At

present, it is very difficult to propagate genetic material of the Cape or African Buffalo in KNP because live animals may not be transported out of the FMD control areas to prevent spreading the disease. Buffalo from the KNP are generally bigger and have a wider horn spread than buffalo occurring in FMDV-free zones of South Africa (7). This increases their value from a safari and game viewing as well as a hunting perspective. The only possible way to currently save and propagate the genetics of the KNP and other FMD positive buffalo is through expensive breeding programs, where disease - free offspring are produced from sero-positive parents. Buffalo calves are separated and isolated from their sero-positive mothers at birth and placed with sero-negative, dairy cow foster mothers. This prevents the transmission of the disease from the buffalo cows to the calves (7). The cost of producing one disease - free buffalo with such a system varies between ZAR 30 000 and ZAR 55 000 (9). The cost of IVP embryos is about ZAR 1 000 per run. A run can be defined as collection of oocytes, *in vitro* maturation (IVM) of the oocytes, *in vitro* fertilisation (IVF) of the oocytes, and *in vitro* culture (IVC) of the zygotes up to the blastocyst stage and can consist of up to 30 oocytes taken through the process together (10). *In vitro* embryo production would be a less expensive way of saving genetic material if embryos can be freed from FMDV and transferred to recipients.

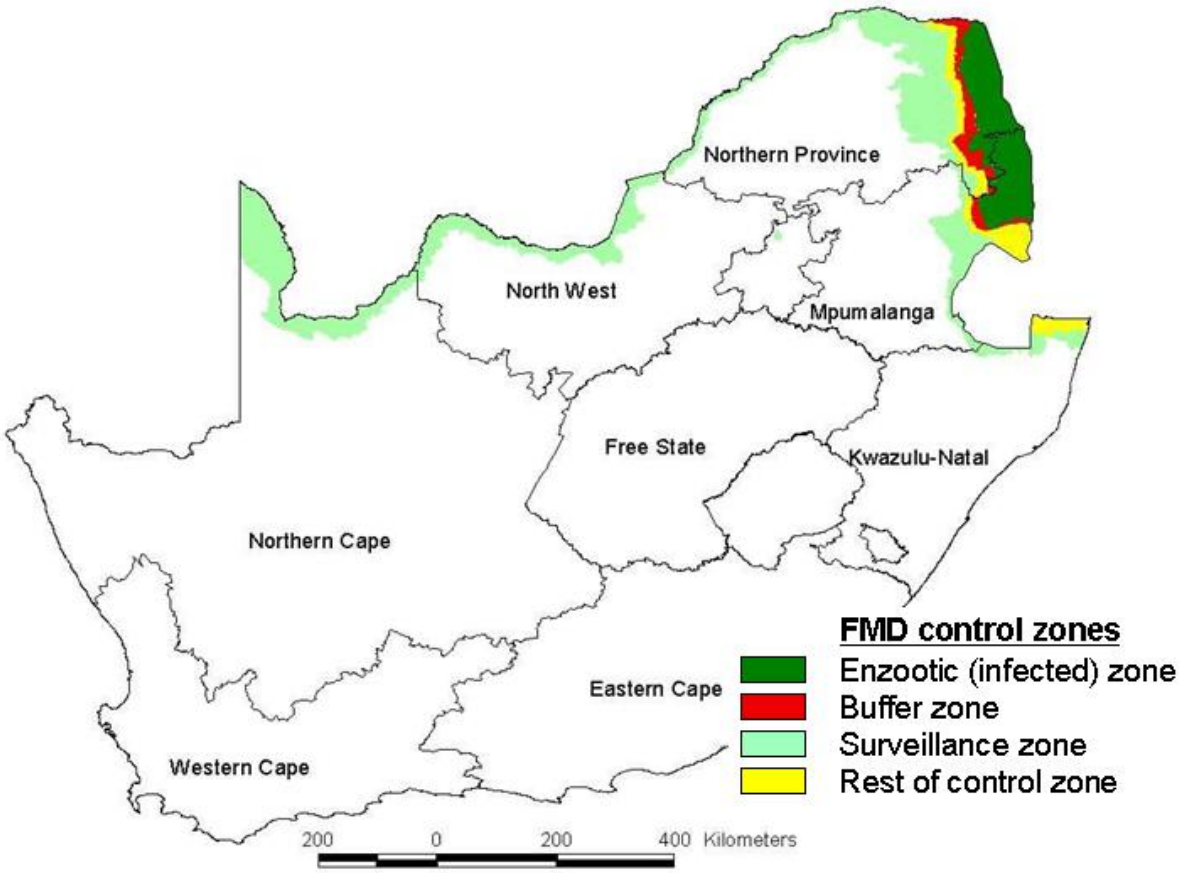

Figure 1.1: Foot and mouth disease (FMD) control zones in South Africa. The enzootic zone encompasses KNP; in the buffer zone vaccination against FMD is practiced; in the surveillance zone vaccination is not practiced, but cattle are inspected every 14 days; in the rest of the control zone cattle are inspected every 28 days (map produced by Sub-Directorate Epidemiology, Directorate Veterinary Services, National Department of Agriculture, South Africa)

The enzootic (infected zone) encompasses the KNP in South Africa. FMDV is harboured in the buffalo population of the park. In the buffer zone, cattle are vaccinated and all cattle are inspected every week. In the surveillance zone, all cattle are inspected every 14 days, but vaccination is not done. In the rest of the FMD control area, all cattle are inspected every 28 days and vaccination is also not done. The rest of South Africa is regarded as free of FMD (11).

1.2 Research Question

The aim of this study was to determine if bovine oocytes exposed to FMDV during IVM and IVF could be freed of FMDV by washing them, or by treating them with acidic organic buffer 2-[N-Morpholino]ethanesulfonic acid (MES). The trial mimics a situation where oocytes or semen, collected from donors in the viraemic phase of FMD are used in an IVP system. The risk of producing FMDV - positive embryos *in vitro* is assessed.

Hypothesis 1: Embryos produced from oocytes co-incubated with FMDV during IVM and IVF can be rendered free from the virus by washing according to International Embryo Transfer Society (IETS) standards.

Hypothesis 2: Embryos produced from oocytes co-incubated with FMDV during IVM and IVF can be rendered free from the virus by acid treatment.

The trial was performed in two consecutive experiments.

1.2.1 Experiment 1: attempt to remove FMDV by washing

The aim of this experiment was to determine if washing cumulus oocyte complexes (COC's) or denuded presumptive zygotes (from here onwards, denuded presumptive zygotes will be referred to as denuded zygotes) ten times according to IETS standards (12), would render them free from FMDV in an *in vitro* embryo production system.

1.2.2 Experiment 2: attempt to remove FMDV by acid exposure

This experiment was done to determine if treating COC's or denuded zygotes with MES could render them free from FMDV in an *in vitro* embryo production system.

2 LITERATURE REVIEW

2.1 Oocyte in the follicle

The oocyte is a round cell of about 20-50 μm in diameter in the primordial follicles (13). During foetal life, a pool of primordial follicles is established in the bovine ovary. Each of these consists of an oocyte arrested in prophase I of meiosis, and a single layer of flattened granulosa cells (14,15). Out of this pool, follicles leave to begin growth. A change in the granulosa cells, from flat to cuboidal, precedes enlargement of the oocyte (15). The follicle grows both by mitotic activity of the granulosa cells and by follicular fluid production. Factors within the follicle maintain the oocyte in meiotic arrest. Purines such as cAMP, hypoxanthine, guanosine and adenosine are amongst others implicated in the maintenance of the meiotic arrest (16). The LH-surge is the signal for the completion of the first meiotic division *in vivo*. At metaphase II, one set of chromosomes is retained within the oocyte, while the other set is extruded as the first polar body. At this stage, division of the oocyte becomes arrested again and will proceed with meiosis II after fertilisation (17). The oocyte in the primordial follicle has no zona pellucida (ZP) and is only surrounded by a thin layer of granulosa cells. As the follicle develops, the surrounding granulosa cells multiply and establish extensive processes towards the oocyte. These processes penetrate into the cytoplasm of the oocyte and may provide nutrients and maternal protein (12,15,18). The ZP is built in the cleft between the oocyte and the granulosa cells. Once the ZP is assembled, the oocyte shrinks to leave the perivitteline space between itself and the ZP and then undergoes the emission of the first polar body as described above (13,19-21). After maturation of the oocyte, most of the cell processes from the granulosa cells retract or disintegrate and the matrix of the ZP closes the channels left by the cytoplasmic processes. The pores that are

detected on the outside of the ZP of matured oocytes probably mark the areas where corona radiata cell - processes entered (18).

The ZP is a shell, which is much thicker than a cell membrane (about 12 μm thick compared to less than 10 nm of the cell membrane) with many functions (22), transparent, porous and glycoproteinacious (15,19,20,23-25). It is essentially a sulphated glycoprotein gel. Three different glycoproteins comprise the ZP. They are termed ZP1 to ZP3 (19,26). Most of the information about the structure and function of the ZP is derived from research on oocytes of mice (26-30). Low power ultrastructural evidence suggests that the ZP has a filamentous construction (31). High power ultrastructural data suggest strongly that the filaments are made up of head-to-tail attachment of globular proteins in single file (32). The filaments are probably made up of repeating dimeric units of ZP2 and ZP3 and are cross-linked by ZP1 (19,20,23,27). Surface glycoproteins ZP3 and ZP2 are species specific and essential during fertilisation. After fertilisation the ZP undergoes the zona block, which prevents other spermatozoa from fertilising the same egg, and it surrounds the early embryo during the first 7 to 10 days, after which it hatches from the zona (15,18,20,33). The glycoproteins of the ZP are produced exclusively by the oocyte, apparently through cleavage of the extra-cellular domains of membrane bound proteins prior to ovulation (13,15,19,34-38).

Follicular fluid is a transudate secreted across the follicular basal membrane. It accumulates in the antrum by coalescence of smaller fluid pockets. The transudate is modified by metabolic activity of the follicular wall and contains specific constituents such as steroids and glycoproteins (15,23,39).

2.2 Oocyte during fertilisation

Ejaculated sperm need a period of incubation in the female genital tract in order to acquire the capacity to fertilize an egg. This process is called capacitation (25,40-42). Capacitation is a process of sperm maturation unique to mammals and it can occur *in vivo* or *in vitro*. Many intracellular and extracellular changes occur during capacitation. Capacitation is accompanied by an increase of membrane fluidity and remodelling of the sperm surface, protein phosphorylation, an increase in intracellular calcium and pH, and membrane hyperpolarization. Spermatozoa following capacitation, also take on a typical hyperactivated movement. This hyperactivated movement not only serves to get the sperm to the oocyte, but it might also be necessary for collision of the sperm cell and the oocyte, enabling adhesion. Only capacitated sperm can undergo the acrosome reaction (25,40-43). Full capacitation of sperm is suppressed in the isthmus storage site of the fallopian tubes and is only completed in the vicinity of the ampullary-isthmic junction at a time close to ovulation (25,34,35,44).

The acrosome reaction is an exocytotic event by which the acrosome fuses with the sperm plasma membrane and exposes its contents to the extracellular environment (26,42,43,45). The typical motility of capacitated sperm and the release of acrosomal enzymes are responsible for the penetration through the ZP. The mechanism of sperm penetration through the ZP is related to proteolysis of the ZP glycoprotein matrix (26,35,45).

The first specific physical interaction between gametes is the binding of sperm to the ZP. This is a key regulatory event in fertilization. Acrosome-intact, capacitated sperm initiate binding to the ZP. The glycoprotein of the ZP that spermatozoa bind to is termed ZP-3. Following the acrosome reaction, which is also mediated by ZP-3, sperm remain bound to the

ZP by ZP-2, another glycoprotein, which binds to the inner acrosomal membrane of the sperm cell. In contrast to ZP-3, which only associates with acrosome intact sperm, ZP-2 only associates with acrosome-reacted sperm (25,26,33-35,43).

After sperm penetrate the ZP, they contact and fuse to the plasmalemma of the egg. Initial changes in the ovum after activation through the fertilising sperm include changes in membrane permeability with a rise in pH, increases in intracellular calcium, cortical granule exostosis and resumption of meiosis II. The function of the cortical granules that are released from beneath the ovum's plasmalemma after sperm fusion and activation is not resolved fully. It appears that they play a role in the hardening of the ZP and inactivation of sperm binding ability mediated by the release of proteases. This modification of the ZP by proteolytic enzymes renders it unable to bind sperm and thus prevents polyspermy (13,15,17,19,25,34-36,45-48).

2.3 Embryo (days 1-7)

The ZP of cleavage stage embryos is an acellular, permeable structure that is spongier on both the inner and outer surfaces than in its centre. Channels left by processes of the corona radiata cells, which made contact with the oocyte previously, traverse it (12,18,23). These channels have a larger diameter on the outer surface of the ZP, decreasing in diameter centripetally (18). The shape of these pores varies and many of them appear elliptical rather than round (39). The ZP of day 7 embryos is smoother and fewer pores are observed by scanning electron microscopy compared to follicular oocytes. This smoother surface and the reduction in porosity may or may not result in differences in zona-pathogen interactions between oocytes and embryos (12,37).

Early bovine blastomeres have rounded contours except where they touch one another. Their plasma membranes are covered with microvilli even in regions of contact at the two- and four- cell stages. By the eight- cell stage the blastomeric plasma membrane is forming focal contacts that are separated by lens-like extracellular spaces. By the 16- cell stage, these focal junctions are lined by electron-dense cytoplasm, but it is difficult to classify the type of junction. This adhesion of blastomeres is calcium dependant and cells can be readily separated in a calcium-free medium *in vitro*. The cytoplasm of the blastomeres during the cleavage stages is characterized by the presence of numerous large vesicles, very large lipidic inclusions, primitive mitochondria containing few peripheral *cristae*, and organelle-free zones in cortical areas of the cytoplasm. The mitochondria are associated with smooth endoplasmic reticulum. Golgi saccules and annulate lamellae are seen in the vicinity of nuclei whereas ribosomes are widespread (23).

Distinct ultrastructural changes occur during compaction of the morula. Amongst others the inner cells of the compacted morula become polygonal, with closely apposed plasma membranes. Consequently cells of a compacted morula are very difficult to separate mechanically (23). There are currently no detailed studies available on *in vivo* compaction of the bovine embryo. It is a very important morphogenic event in embryonic development and it is well documented in the mouse in which it happens at the eight-cell stage. The cells become polarized as tight junctions form between them, thereby segregating inner cells from outer cells, and they begin to communicate with each other through the intercellular structure (17). The polarization of cells plays a part in the formation of the blastocyst (23).

2.3.1 Comparison of *in vitro*- and *in vivo*-derived bovine embryos

There are some obvious differences between day-7 embryos produced *in vivo* in the cow and those produced *in vitro*. Obvious differences are the compacted appearance of *in vitro*- vs. *in vivo*-derived embryos and the fact that IVP morulae and blastocysts often have fewer cells than those collected from a cows uterus (12,38).

These obvious differences are well known, but important subtle differences that are not widely recognized by those interested in embryo-pathogen interactions relate to the structure of the ZP (38). Using monoclonal antibodies produced against ZP proteins from day 7 *in vivo*-derived embryos, it was confirmed that there are differences between the ZP of follicular oocytes, such as would be used in the *in vitro* production of embryos, recently ovulated oocytes, and the ZP of developed embryos collected from the cow's uterus (38,49). Using scanning electron microscopy topographical differences can be seen as well (17,37,38).

The ZP of IVM oocytes appear spongier and have more numerous pores compared to IVP zygotes when examined with scanning electron microscopy (18). The mean outside diameter of the pores in the ZP of bovine IVP oocytes is 182±69 nm and that of IVP morula is 155±71 nm (18). This might have some relevance to the interaction of the ZP with pathogens. Fluorescent microspheres with a diameter of 40 nm are able to penetrate halfway through the pores of the ZP, but are unable to fully traverse the ZP of bovine IVP oocytes or embryos (18).

Data on the diameter of processes of the corona radiata cells extending through the ZP of bovine oocytes *in vivo* are difficult to find. In the pig and the rat they seem to be about 50-

100 nm in diameter. Within 2-6 hours after ovulation in livestock species the corona radiata cells are shed from the ZP and the matrix of the ZP obliterates the channels (12).

Whether the differences between the ZP of *in vivo*- and *in vitro*-derived bovine embryos are accompanied by differences in their capabilities to resist adherence or penetration by infectious agents has not been investigated adequately (12,17,38). However, IVP embryos that were exposed to FMDV could not be freed of the virus by washing them ten times according to IETS standards, as was the case with *in vivo*-derived embryos (12,49). Also, when oocytes were exposed to bovine herpes virus-1 (BHV-1) during IVM and IVF, neither oocytes nor embryos could be washed clean of the virus (50,51). In contrast to this, *in vivo* -produced embryos can be rendered free from BHV-1 by washing and trypsin treatment. It is not known if trypsin treatment is effective in inactivating BHV-1 associated with zona-intact IVF embryos (12). It has also been reported that conventional washing procedures for *in vivo*- produced embryos to remove bovine virus diarrhoea virus (BVDV) might not be adequate for removal of the virus from IVP embryos (52). Thus, there is good reason to believe that differences exist in the capabilities to resist adherence or penetration by viruses between *in vitro* and *in vivo* embryos.

2.4 Characteristics of Foot-and-mouth disease virus

2.4.1 Economic impact of Foot-and-mouth-disease virus

Foot-and-mouth disease is an extremely contagious viral disease of ruminants and pigs. Economically it is the most important disease of livestock in southern Africa and probably in the world (2,3,5,17). The economical implications in the event of an outbreak are

devastating. Not only are huge losses incurred by slaughtering of positive and adjoining herds or by vaccination campaigns to contain the disease in the face of an outbreak, but countries free of the disease also ban imports of any agricultural products from a country where a case of FMD occurred. Large amounts of money are spent annually to prevent importing infected material into South Africa, in order to retain South Africa's status as FMDV free and as an exporter of agricultural products, and to control and prevent the disease from spreading in the areas surrounding the park (1,3).

2.4.2 Structure of Foot-and-mouth-disease virus

The FMDV belongs to the family *Picornaviridae* and is a member of the genus *Aphthovirus.* It is a non-enveloped, single stranded RNA virus. There are seven serological types (A, O, C, SAT-1, SAT-2, SAT-3, and Asia 1). Due to high rates of mutation, many subtypes exist (1-3,53). Just like other picornaviruses, FMDV has a dry diameter of 27-28 nm, is spherical in shape and exhibits icosahedral symmetry. The icosahedral capsid is a polymere composed of 60 protomeres, the majority of which contain one molecule of each of the three structural proteins, VP1 – VP3. Five of these protomeres will produce a pentamere and 12 pentameres conform the complete capsid (2). A few protomeres within each capsid are immature and contain VP0 instead of VP2 and VP4. VP0 is the uncleaved precursor of VP2 and VP4. VP1-3 is partly exposed at the surface, while VP4 is internal (1,2,53-55). The virion consists of approximately 70% protein and 30% RNA, as well as a small quantity of lipid. Unlike other picornaviruses, FMDV does not seem to possess any major canyons or pits on the surface of the virus (53,56). Figure 2.1 shows a representation of the FMD virion at 2.9Å resolution indicating the relative position of the four structural proteins and a pentamere comprising five protomeres with the relative positions of the four structural proteins.

Residues 141 – 160 of VP1 have been identified as the major neutralizing site of the virus (53,56). High levels of neutralizing antibody are produced by peptides containing this sequence (56). Sequences from the C-terminal region of VP1 can also trigger the production of antibodies, but to a much lower level (56).

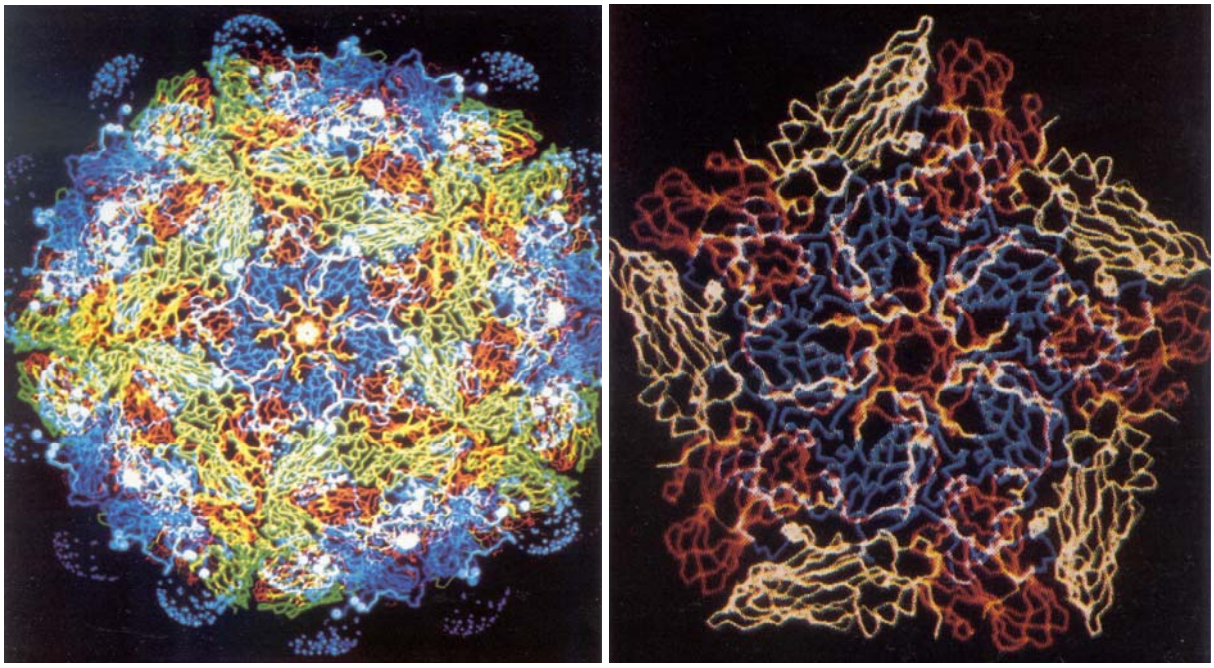

Figure 2.1: Left-Representation of the Foot-and-mouth disease virion at 2.9Å resolution. Right-A pentamere comprising 5 protomeres showing the relative positions of the four structural proteins. The view of the five fold axis is from the outside. Colour coding: blue = VP1; Green = VP2; Red = VP3; Yellow = VP4 (53).

The cell-attachment site on the virus involves the loop 133 - 158 of VP1 (FMDV loop) and the C-terminal region of VP1 (53). Attachment to a susceptible cell can be prevented by all antibodies known to bind to this site, by proteolysis of this loop or the C-terminal region of VP1, and by peptides containing the highly conserved sequence Arg-Gly-Asp at residues 145 -147 (53,56). Attachment of the virus to cells is dependent on calcium ions (53,56).

The FMDV is pH sensitive (1,56). At pH 6 the FMDV is inactivated at a rate of 90% per minute, while at pH 5, it is 90% per second. The virus disintegrates into its subunits in mildly acidic solutions. Although this liberates infectious RNA, it is usually rapidly inactivated by environmental ribonucleases (1,53).

2.4.3 Transmission of Foot-and-mouth disease virus

Measurable quantities of FMDV are present in animal secretions and excretions for less than two weeks after infection; however, oesophageo-pharyngeal secretions have been reported to contain FMDV as long as a few months or even years (1,4,57). In viraemic animals, FMDV is present in all physiological body fluids. Quantities of virus in different body fluids vary, but appreciable amounts do occur in the urogenital tract (1,57).

Secretion of significant amounts of virus may occur up to four days or longer before the appearance of lesions. Animals that are apparently healthy (although viraemic) can thus be an important source of the virus (1).

Infection via the oral route in cattle requires at least 10 000 times as much virus as via the respiratory route. It is therefore believed that the usual portal of entry is via the respiratory tract. Initial viral replication takes place in the mucosa and lymphoid tissue of the tonsillar region. These regions are exposed directly to the virus, which enters the body either by inspiration or orally, as well as indirectly from virus cleared by the mucocilliary mechanisms from the nasal passages and the lungs, and from ingested virus regurgitated during rumination (1). The liberation of virus into the efferent lymphatic system results in an initial viraemia whereby virus reaches a wide variety of organs and tissues (1,4).

2.4.4 Adhesion of Foot-and-mouth disease virus

Attachment of FMDV to cells is effected by electrostatic affinity between specific virus receptors and their binding sites in the plasmalemma of the cell. This viral attachment is largely dependant on the function of the VP1 loop. The configuration and chemical composition of these attachment sites on the plasmalemma are unknown, but they must have some other biological function as attachment of the virus to the cell in itself can be damaging to the host cell (1,56).

2.4.5 Internalisation of Foot-and-mouth disease virus

Entrance of FMDV occurs via pinocytosis after attachment to the host cell. VP4 is lost during this process. After internalisation viral RNA is liberated from the capsid into the cytoplasm. The viral RNA is translated into a large polyprotein, which is sequentially cleaved by viral proteases. This cleavage gives rise to the different structural and non-structural proteins (1).

2.4.6 Replication of Foot-and-mouth disease virus

Progeny RNA produced by a complementary negative - sense strand, occurs on the smooth endoplasmic reticulum and is initially extremely rapid, generating many positive - sense copies (58).

The virus inhibits cellular protein and RNA synthesis while it's own RNA synthesis proceeds rapidly in the cytoplasm. The RNA strands direct synthesis of a polyprotein, which is cleaved into individual proteins as synthesis progresses. The structural proteins are assembled to form

new virions. These virions are released through infection-mediated disintegration of the host cell (58).

2.5 Foot-and-mouth disease virus and embryo transfer

2.5.1 Association of Foot-and-mouth disease virus with the bovine embryo

High virus concentrations ($10^{1.2}$ to $10^{4.4}$ plaque forming units (pfu)/ml) were found in the follicular fluid and the ovarian tissue ($10^{<2.2}$ to $10^{7.1}$ pfu/sample) of infected, viraemic cows (59). On the contrary, low levels of virus were found in flushing fluid used to collect embryos from viraemic animals. When convalescent cows were killed within six weeks after the disease and their ovaries examined for the presence of the virus, no infectious agents could be demonstrated. This indicates that FMDV does not persist in the ovaries of infected cows (59).

There has been some research on the association of the virus with *in vivo*-derived embryos and this research shows that the risk of disease transmission by this route is extremely small (12,24,49,59,60). When ZP–intact embryos were collected at day 6 post-oestrus from slaughtered cows, and then exposed to 10^6 pfu/ml of FMDV for 2 to 18 hours in culture medium at 37°C, no viral infectivity could be demonstrated in the embryos if these embryos were washed according to IETS standards after exposing them to the virus (12). No cytopathic effect could be observed on bovine kidney (BK)-cell culture or by inoculating into the tongue of steers, after washing the embryos (12,24). Similarly, if embryos were collected from viraemic donors and washed ten times according to IETS standards (12), no viral infectivity could be demonstrated in BK-cell culture or by inoculating into the tongue of

steers (12,59). When ZP-intact embryos, collected from viraemic donor animals, were washed ten times according to IETS standards and then transferred to recipient cows, none of the cows seroconverted or developed FMD. None of the calves born to these cows were seropositive or developed the disease (12,60). It seems that the ZP protects the embryos from becoming infected with FMDV, because embryos without an intact ZP cannot be cleaned of the virus by washing them according to IETS standards (24). The IETS manual is very clear about the fact that embryos are only regarded as safe when they have an intact ZP, are free of adherent material and are washed 10 times (12). It is not known if the virus gets trapped between surface folds of the embryo or if it infects the embryonic cells *per se* when an intact ZP is not present. The *in-vitro* development of embryos without an intact ZP does not seem altered and therefore it is assumed that the virus does not infect the embryonic cells, but rather gets trapped in surface folds because attachment of the virus alone can be damaging to the host cells (1,24). Also, when day 6 (ZP-intact) or day 11 (ZP-free) bovine embryos are collected from donor cattle and incubated with FMDV, no obvious difference can be noted by light microscopy in the *in vitro* development as compared to uninfected embryos (24).

It has been shown that the ZP of bovine embryos resists adherence and penetration by many pathogens including FMDV (33,39,61). The precise mechanism by which FMDV associates with the bovine ZP is not known. It is known, however, that most infectious agents that do associate with embryos adhere to the ZP rather than penetrating it. Methods to dislodge virus particles, in addition to washing ten times according to IETS standards include, treating with trypsin and treating with glycosidases or neuraminidase to dislodge virus (12). A reduced pH for a short period of time would probably not affect the embryo itself inside of the ZP, but it would likely dislodge or even lyse virus particles (39). Singh et al (61) indicated that enveloped viruses are susceptible to inactivation/removal by treatment with trypsin, whereas

non-enveloped viruses are not. However, it has been shown that trypsin cleaves the loop 133-158 of VP1 of the FMDV, which renders the virus incapable of binding to cells (1).

Very little work has been done on the association of FMDV with IVP embryos. *In vitro*- and *in vivo*-derived embryos are different in structure and there are indications that IVP embryos carry a greater risk of transmitting diseases (12,17,37,38,49,62). This is probably due to differences in the structure of the ZP (38). No research has been done on the association of FMDV with bovine embryos during IVM and IVF. However, it has been reported that day 7 IVP embryos, exposed to FMDV, could not be freed of the virus by washing according to protocols recommended by IETS (12,38). Exposing oocytes to FMDV during IVM and IVF may provide a more realistic model to judge if oocytes collected from infected donor cattle pose a risk of transmitting FMDV.

There are no research results in the literature describing the rate of survival of the FMDV in the different IVC media. It is therefore not known if conditions under which embryos are cultured and the media in which this is done in the laboratory, will support survival of the virus. It is however likely that the *in vitro* media will support the survival of the virus, because of the physiological pH and osmolarity of the different media.

The pores on the surface of the ZP of IVM bovine oocytes (182±69 nm) and of IVP morulae (155±71 nm) would seem to be large enough to allow entry of the FMDV (dry diameter of 27-28 nm) (2,18). The diameter of the pores decreases centripally and it is uncertain if the FMDV would be able to pass all the way through the ZP. Only one virus has thus far been shown to be able to penetrate the ZP of farm animals. This is the porcine parvovirus with a diameter of 20 nm; it was detected in ZP – intact, *in vivo*-derived porcine

embryos after incubation with the virus (63). In mice, Mengo encephalitis virus with a diameter of 27-28 nm (similar diameter to FMDV), has been shown to penetrate the ZP of 2-cell embryos and morulae (64,65). Viral penetration presumably occurs along channels left in the ZP when the granulosa cell - processes are withdrawn. The ZP of mouse embryos is however much thinner (about 5 μm) compared to that of the bovine ZP (approximately 10-15 μm). This may be relevant to its function as a barrier against pathogens (12).

3 MATERIALS AND METHODS

3.1 Introduction

Cattle oocytes were matured and fertilised *in vitro*, and the resulting embryos cultured, according to standard procedures used in the Section of Reproduction, Onderstepoort and are described in detail below. The embryo yield in the IVF laboratory of the Section of Reproduction compares favourably with the yield of other laboratories internationally (66).

FMDV was added to the oocytes during IVM and IVF to mimic a situation where oocytes are maturing in FMDV contaminated follicular fluid or where contaminated semen would be used. Attempts were made to rid the oocytes or zygotes of virus before the blastocyst stage by washing according to IETS standards (12) or by acid treatment.

3.2 Experimental design

The study was split in two separate experiments. In experiment 1 (E1), an attempt was made to wash FMDV from zygotes/embryos according to the IETS washing procedure. In experiment 2 (E2), an attempt was made to remove FMDV from oocytes or embryos by exposing them to the organic acid MES.

3.2.1 Experiment 1: attempt to remove FMDV from embryos by washing

3.2.1.1 Summary of experiment 1

For experiment 1 COC's were collected through aspiration of slaughterhouse ovaries and were placed at random into one of the following four groups: A control group (E1G1) which was not exposed to FMDV and was not washed prior to FMDV isolation. Group 2 (E1G2) was exposed to FMDV during IVM to mimic a situation where oocytes would be collected from a viraemic cow with contaminated follicular fluid. FMDV was not added again during IVF in this group and the denuded zygotes were washed after IVF to assess if they could be cleared from FMDV by washing. Group 3 (E1G3) were treated the same as E1G2 except that denuded zygotes were not washed after IVF, but instead the zygotes were submitted to IVC and the resulting embryos were washed after IVC. Group 4 (E1G4) was treated the same as E1G3 except that FMDV was again added during IVF to mimic a situation where contaminated semen would be used in addition to FMDV contaminated oocytes.

3.2.1.2 Detailed description of groups in experiment 1

One group of 250 oocytes (E1G1) and three groups of 270 oocytes (E1G2 - 4) were created from the 1060 COC's collected through aspiration of 200 slaughterhouse ovaries. Each group was further subdivided into batches (batches were replicates of the treatments for each group) and were treated exactly as follows:

<u>Group 1:</u>

Group 1 (E1G1) was the control group with 250 COC's. COC's in this group were not exposed to FMDV. The embryos that were produced were not washed ten times before detection of FMDV was attempted to act as a negative control. Three replicates of this group

were done with two batches of 100 oocytes each and one batch of 50 oocytes. The oocytes were matured in 3 ml of IVM fluid in 3 small petri dishes as described in chapter 3.3. Fertilisation of the COC's was performed as described in chapter 3.3. Cleavage of the embryos was assessed on day 4 after fertilisation. Detection of FMDV was attempted in the resulting embryos at day 7 after fertilisation with polymerase chain reaction (PCR) and PK cell culture (PKCC) to detect if there was contamination with FMDV (Figure 3.1). Samples of IVC medium were also analysed for the presence of FMDV. Standard operating procedures for PCR and PKCC are described in Appendix B (Chapter 8). The blastocyst rate was also recorded for comparison between the control and different treatment groups.

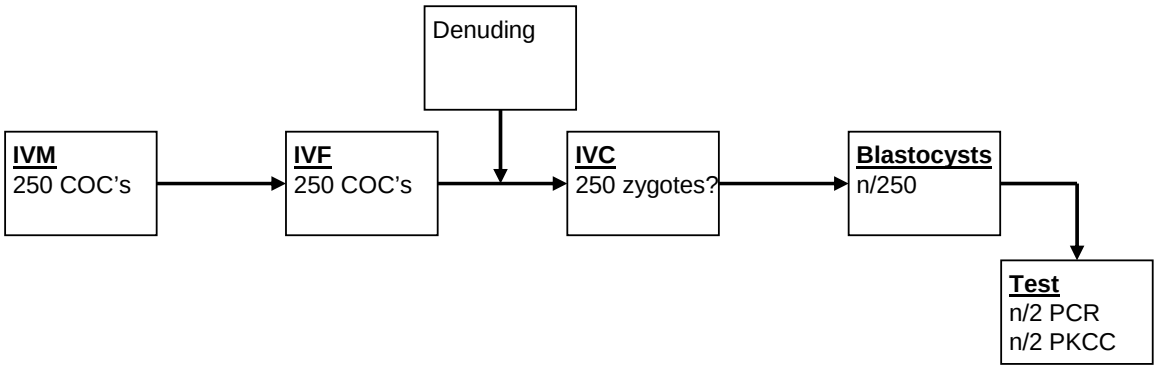

Figure 3.1: Experiment 1, Group 1, control, (E1G1). Flow diagram of experimental procedures. IVM, IVF and IVC were done in the standard way. No FMDV was added in this group. Detection of FMDV was attempted in the blastocysts to control for contamination of the media.

Group 2:

Group 2 (E1G2) consisted of 270 COC's. Again three replicates of this group were done with two batches of 100 oocytes and one batch of 70 oocytes. The oocytes were matured in small petri dishes containing 3 ml IVM fluid to which 200 µl of the virus suspension was added.

The COC's were matured and co-incubated with FMDV for a total of 24 hours. Ten oocytes, selected randomly from the three petri dishes, together with their cumulus cells were removed from this group after IVM and analyzed for the presence of FMDV to assess if the maturation medium affected the survival of the virus (five COC's were transferred to 200 µl phosphate buffered saline (PBS) for detection of the virus with PCR and five COC's were transferred to 1 ml PBS for detection of FMDV with PKCC).

The remaining 260 oocytes of E1G2 were fertilised in their respective batches without adding any FMDV (Figure 3.2). After IVF, 10 presumptive zygotes were removed to assess if any FMDV was still present (assuming that IVM did not already destroy the virus) after 20 hours in the fertilisation medium. (Five zygotes were transferred to 200 µl PBS for detection of the virus with PCR and five zygotes were transferred to 1 ml PBS for detection of FMDV with PKCC).

The remaining 250 zygotes (in three separate batches) were denuded and washed ten times with FERT WASH medium (chapter 7.4) in accordance with IETS standards (12). Half (125) of the zygotes were suspended in 1 ml PBS for virus detection using PKCC and the remaining half was suspended in 200 µl PBS for virus detection using PCR. Samples of the cumulus cells were also handled in a similar way for detection of FMDV with PKCC and PCR.

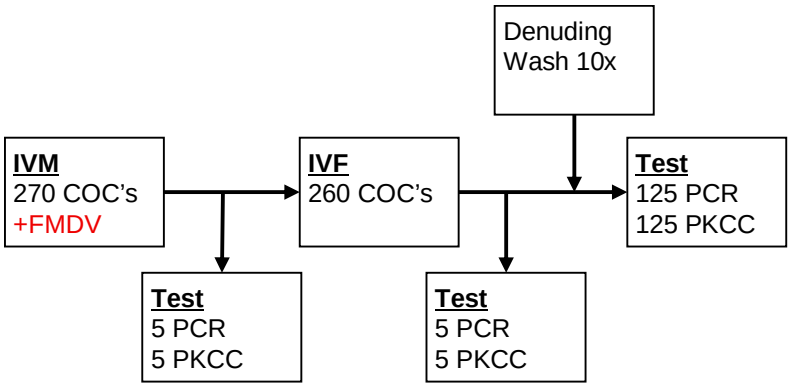

Figure 3.2: Experiment 1, Group 2, (E1G2). Flow diagram of experimental procedures. FMDV was added during IVM, but not during IVF. Denuded structures were washed after IVF and detection of FMDV was attempted in/on them.

Group 3:

Group 3 (E1G3) consisted of 270 oocytes. Three replicates of this group were done with two batches of 100 oocytes and one batch of 70 oocytes. This group was treated exactly the same as the oocytes in E1G2 up to denuding following IVF. After denuding they were submitted to IVC (Figure 3.3).

Cleavage of the zygotes was assessed on day 4 after fertilisation. On day 7, the blastocyst rate was recorded and the resulting embryos were washed ten times with FERT WASH (chapter 7.4) according to IETS standards (12). Half of the embryos were suspended in 1 ml PBS for virus detection using PKCC and the remaining half was suspended in 200 µl PBS for virus detection using PCR.

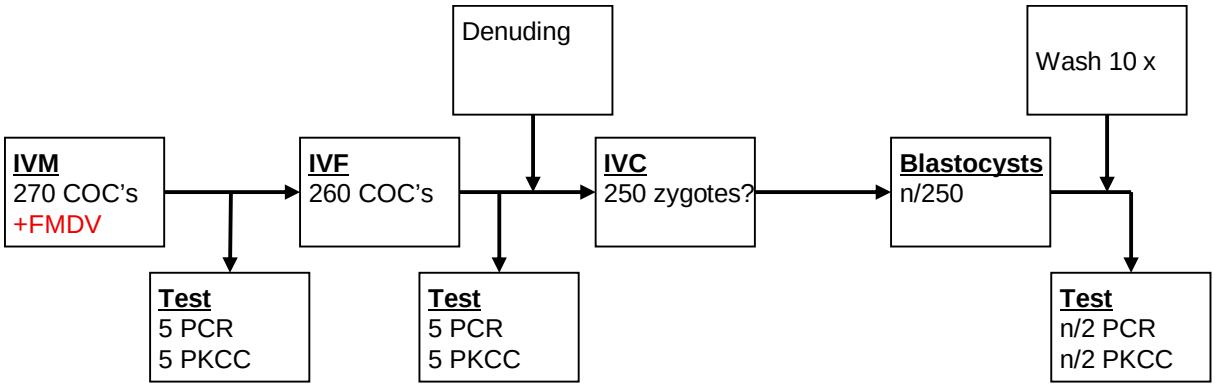

Figure 3.3: Experiment 1, Group 3, (E1G3). Flow diagram of experimental procedures. FMDV was added during IVM, but not during IVF. Washing was done after IVC and detection of FMDV was attempted in the resulting embryos.

Group 4:

The 270 oocytes of group 4 (E1G4) were divided into 3 batches as in E1G3 (for 3 replicates) and treated as the oocytes of E1G3, but FMDV was added to this group during IVF (Figure 3.4). FMDV was thus added both during IVM and IVF. It was not important during these trials to be able to assess if FMDV in the resulting embryos originated from IVM or IVF (or both), but rather to assess if we could rid the embryos of FMDV by washing regardless of the origin of the virus. This group mimicked the worst-case scenario where both infected oocytes and infected semen would be used in an IVP system.

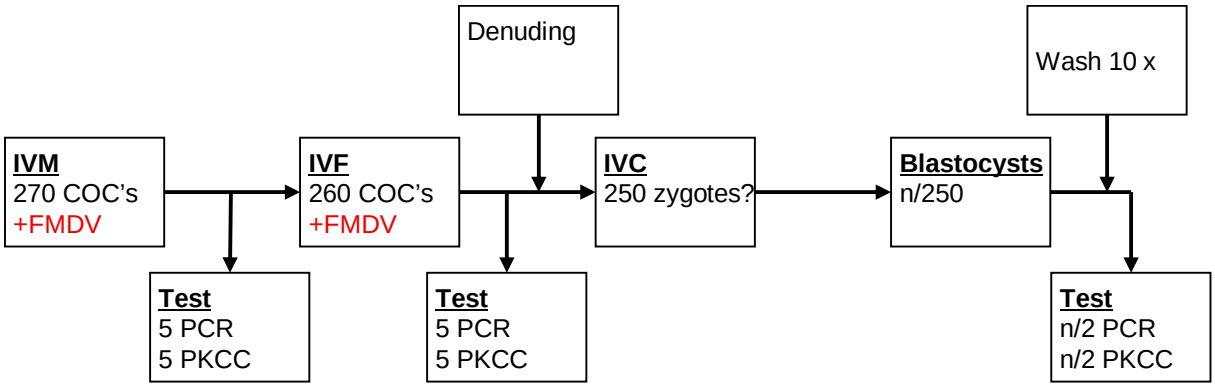

Figure 3.4: Experiment 1, Group 4, (E1G4). Flow diagram of experimental procedures. FMDV was added during IVM and IVF. Washing was done after IVC and FMDV detection was attempted in the resulting embryos.

3.2.2 Experiment 2: attempt to remove FMDV from oocytes or embryos by MES exposure

3.2.2.1 Summary of experiment 2

COC's were collected from slaughterhouse ovaries and randomly assigned to one of six groups as follows: Group 1 (E2G1) was the control group. The control group was not exposed to FMDV and washing or MES treatment was not done in this group before detection of FMDV was attempted to act as negative control. In group 2 (E2G2) oocytes were exposed to FMDV during IVM and IVF to mimic a situation where oocytes would be collected from viraemic cows with FMDV contaminated follicular fluid and where semen would be contaminated with FMDV. Denuded zygotes were treated with MES after IVF to assess if they can be cleared of FMDV. Group 3 (E2G3) was treated the same as E2G2 except that denuded zygotes were not treated with MES after IVF. Instead, the zygotes were submitted to IVC and MES treatment was done after IVC to test if embryos are free from FMDV. In

group 4 (E2G4) COC's were co-incubated with FMDV during IVM. The COC's were treated with MES after IVM to assess if it is possible to clean COC's from FMDV. In group 5 (E2G5) FMDV was added during IVM and during IVF. MES treatment was also done after IVM and after IVF to assess if two MES treatments are sufficient to rid denuded zygotes from FMDV. Group 6 (E2G6) was treated the same as E2G5 except that denuded zygotes were not tested for the presence of virus. Instead, zygotes were submitted to IVC and tested thereafter. This group was done to test if two MES treatments are sufficient to get rid of FMDV as in group 5, but also to see if more than one MES treatment is detrimental to the development of blastocysts in our system by comparing blastocyst rates between the groups. In none of the treatment groups of experiment 2 were washing performed before MES treatment.

3.2.2.2 Detailed description of groups in experiment 2

Two groups of 320 oocytes (E2G2-3) and four groups of 300 oocytes (E2G1, E2G4 - 6) were compiled from the 1840 COC's collected through aspiration of 300 slaughterhouse ovaries. Each group was further subdivided into batches (as in experiment 1, batches were replicates of the treatments for each group) and were treated exactly as follows:

Group 1:

In Group 1 (E2G1, control) 300 COC's were submitted to IVM, IVF and IVC. Three replicates of this group were done in batches of 100 COC's each. Half of the blastocysts harvested on day 7 of IVC were tested for the presence of FMDV with PCR and the other half with PKCC. MES treatment was not done in this group before testing for the presence of FMDV to act as negative control (Figure 3.5).

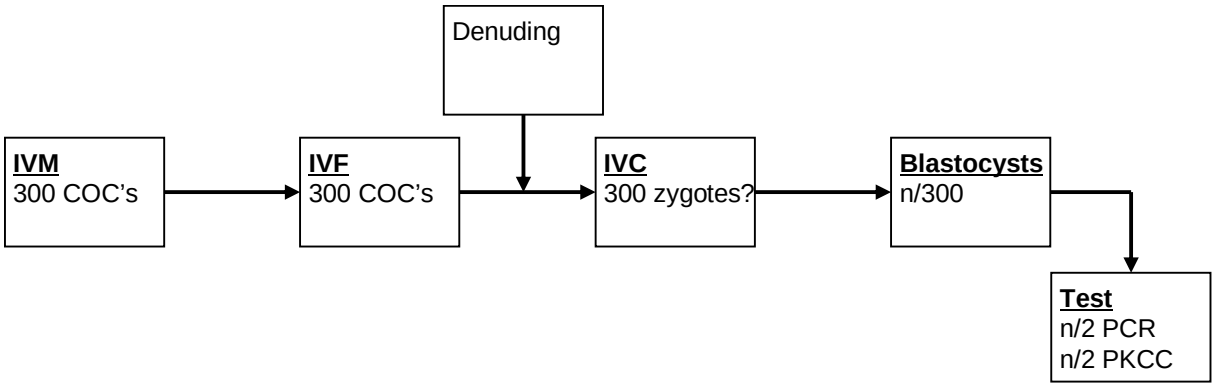

Figure 3.5: Experiment 2, Group 1, control, (E2G1). Flow diagram of experimental procedures. IVM, IVF and IVC were done in the standard way. No FMDV was added in this group. Detection of FMDV was attempted in the blastocysts to control for contamination of the media.

Group 2:

In Group 2 (E2G2; minimal MES treatment), 320 COC's were exposed to FMDV during IVM and IVF. Three replicates of this group were done in batches of 100 – 110 COC's. During IVM 200 µl of FMDV-suspension was added to each batch of COC's. In addition, 10 µl of FMDV-suspension was added to each droplet during IVF. Ten COC's were removed after IVM, and another ten after IVF and denuding, to test for the presence of FMDV. The remaining 300 zygotes were exposed to MES after denuding and before being tested for the presence of FMDV (Figure 3.6). The protocol for MES treatment is described under experimental procedures (chapter3.3).

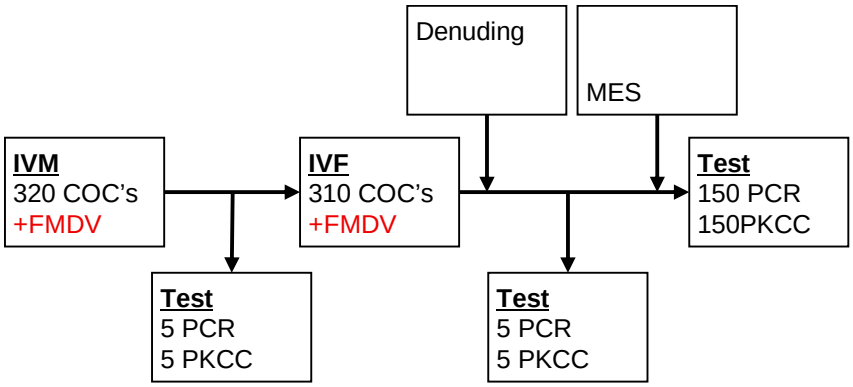

Figure 3.6: Experiment 2, Group 2, (E2G2; minimal MES treatment). Flow diagram of experimental procedures. FMDV was added during IVM and IVF. Denuded structures were submitted to MES treatment after IVF. The presumptive zygotes were tested for the presence of FMDV.

Group 3:

Group 3 (E2G3; minimal MES treatment) consisted of 320 oocytes. Three replicates of this group were done in batches of 100 – 110 COC's. COC's in this group were treated exactly as for those described in group E2G2. The only difference was that detection of FMDV was not attempted in the 300 zygotes after denuding and treatment with MES, but the zygotes were cultured for 7 days after MES treatment before being tested for the presence of FMDV (Figure 3.7).

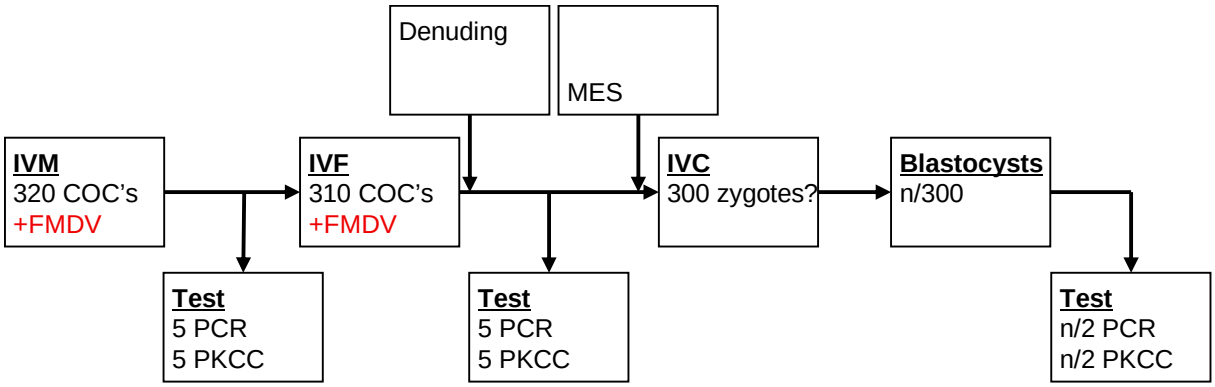

Figure 3.7: Experiment 2, Group 3, (E2G3; minimal MES treatment). Flow diagram of experimental procedures. FMDV was added during IVM and IVF. Denuded structures were treated with MES after IVF. Presumptive zygotes were submitted to IVC and detection of FMDV was attempted in the resulting embryos.

Group 4:

Group 4 (E2G4) consisted of 300 COC's that were placed in IVM together with FMDV (Figure 3.8). Three replicates of this group were done in batches of 100 COC's. After IVM, the COC's were treated with MES as described for the denuded zygotes in Groups 2 and 3 before detection of FMDV was attempted.

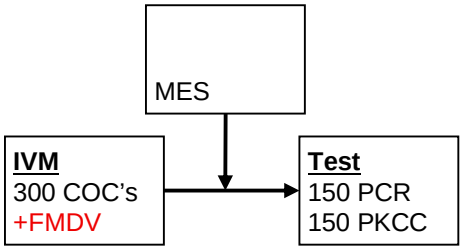

Figure 3.8: Experiment 2, Group 4, (E2G4). Flow diagram of experimental procedures. FMDV was added during IVM. COC's were treated with MES after IVM and detection of FMDV was attempted.

Group 5:

Group 5 (E2G5; maximal MES treatment) consisted of 300 COC's that were placed in IVM and IVF in the presence of FMDV. Three replicates of this group were done in batches of 100 COC's. After IVM, the COC's were treated with MES as described for those in Group 4. After IVF and denuding, the zygotes were again treated with MES before detection of FMDV was attempted to assess if 2 treatments with MES is effective to remove FMDV (Figure 3.9).

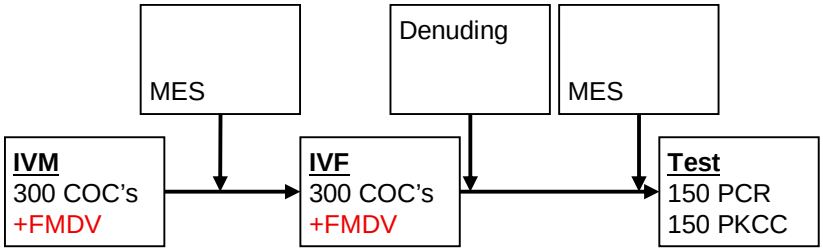

Figure 3.9: Experiment 2, Group 5, (E2G5; maximal MES treatment). Flow diagram of experimental procedures. FMDV was added during IVM and IVF. Denuded structures were treated with MES after IVF and detection of FMDV was done in the presumptive zygotes.

Group 6:

Group 6 (E2G6; maximal MES treatment) consisted of 300 COC's that were treated exactly as for those described in Group 5 except that detection of FMDV was not attempted in the 300 zygotes after denuding and treatment with MES, but the zygotes were submitted to IVC to assess if 2 MES treatments were detrimental to the development of embryos. Again, three replicates of this group were done with batches of 100 COC's. Detection of FMDV was attempted in the resulting embryos on day 7 (Figure 3.10).

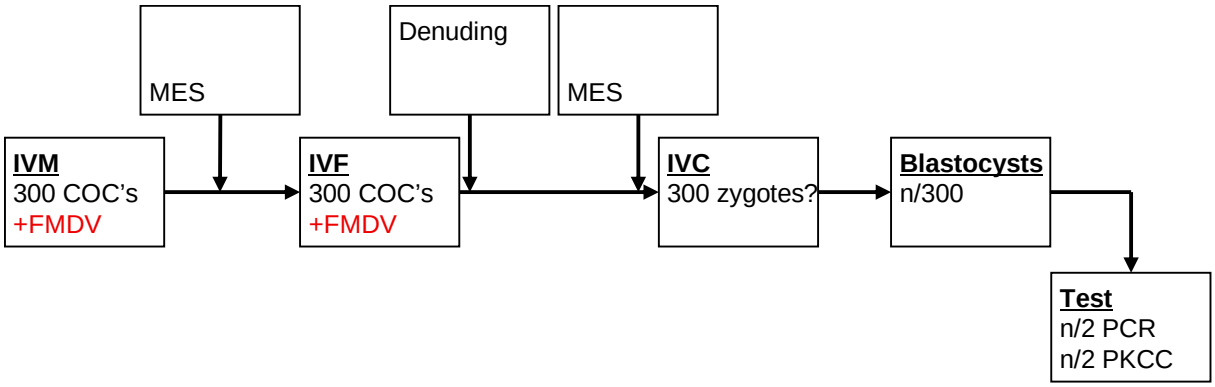

Figure 3.10: Experiment 2, Group 6, (E2G6; maximal MES treatment). Flow diagram of experimental procedures. FMDV was added during IVM and IVF. Denuded structures were treated with MES after IVF and the presumptive zygotes were submitted to IVC. Detection of FMDV was attempted in the resulting embryos.

3.2.3 Statistical analyses

No statistical analysis was performed to test whether or not FMDV could be removed from COC's, denuded structures or embryos. This was measured as a categorical value; i.e. either there was FMDV or there was none.

Comparing cleavage and blastocyst rate between treatment and control groups tested the effect of washing or MES treatment on embryonic development. A χ^2-test at $p<0.05$ was used to test for differences with the statistical program NCSS 2001[1].

The trials were designed to detect a difference in development rate of 20%. With 200 oocytes a 20% difference in either cleavage or blastocyst rate (degrees of freedom = 1) can be

[1] NCSS Statistical Software, 329 North 1000 East, Kaysville, Utah 84037

detected with $p<0.05$ and a power of 0.8; with 263 to 300 oocytes the power can be increased to 0.9.

A χ^2-test at $p<0.05$ ($n>241$; degrees of freedom = 2; power = 0.8; detection limit 20%) was used to test for differences among the 3 replicates of each group.

3.3 Experimental procedures

3.3.1 Collection of oocytes

The exact number of oocytes used during the experiments was limited by the number of ovaries available for collection. It was attempted to collect 100 COC's for each one of the 3 replicates of each group. Bovine ovaries (from cows negative for FMDV) were harvested in a slaughterhouse within 30 minutes of slaughter of cows and stored in 2.5 l thermos jugs containing PBS (chapter 7.1) at 32˚C. The ovaries were delivered to the *in vitro* laboratory at Onderstepoort[2] within 6 hours of collection. The temperature of the PBS was taken before aspiration of the follicles; it was maintained between 27˚C and 30˚C. Follicles of 5 mm diameter or less were aspirated using a 10 ml Syringe (B Braun[3] 4616103F) and an 18 G needle (Terumo[4] NN-1838S). Follicular fluid was collected in sterile 15 ml centrifuge vials (Kima [5] 17020) containing 2 ml tissue culture medium (TCM199-H, Sigma [6] H2520) supplemented with 0.3% bovine serum albumin (Sigma A9418) and 25µg/ml Gentamycin (Sigma G1264). The tube containing the medium and follicular fluid was kept in a waterbath

[2] Faculty of Veterinary Science, University of Pretoria, P/bag X04, Onderstepoort, 0110, South Africa
[3] B Braun Medical Inc.,901 Marcon BLVD, Allentown, PA 181039341, U.S.A.
[4] Terumo Europe, Hauptsrasse 87, D-65760 Eschborn, Germany
[5] Kima, 35020 Arzergrande (PD), Italy
[6] Sigma-Aldrich S.A. (Pty) Ltd, Aston Manor 1636, Gauteng, South Africa

at 32˚C until oocyte searching. COC's were located (10x magnification) and evaluated (80x magnification) in a petri dish (Corning[7] 430591) containing 10 ml culture medium under a stereomicroscope and were selected on the basis of visual morphology and number of cumulus cells. Only oocytes with at least one complete layer of cumulus cells and homogenous appearing protoplasm were selected and used for the completion of the experiments (67). The selected COC's were washed twice in TCM 199-H to remove follicular debris. Groups of 100 COC's were then transferred to 2 ml IVM medium (chapter 7.7) (68,69). The steer serum used in the medium was collected from a FMDV-free steer housed at the Faculty of Veterinary Science, Onderstepoort. The COC's were kept in 5 ml round bevel cryogenic vials (Corning 430499) in an incubator at 38˚C with an atmosphere of 5% CO_2 and 100% humidity before being transported to the Exotic Diseases Division of the Onderstepoort Veterinary Institute[8] where the experiments with the FMDV were performed. While the COC's were in the incubator, the lids of the cryogenic vials were not tightly closed to ensure that the gas inside the vials had the same composition as that in the incubator. The vials were left inside the incubator for at least one hour to allow time for the gas inside of the vials to equilibrate with the gas in the incubator. When the vials containing the COC's were removed from the incubator for transport to the Exotic Diseases Division, the lids were tightly closed to prevent any gas exchange with the environment. The Exotic Diseases Division, where the experiments were conducted, is located approximately 2 kilometres from the Onderstepoort Veterinary Faculty where the ovaries were aspirated and the oocytes selected. The vials containing the oocytes were transported to the Exotic Diseases Laboratory in a portable incubator (Minitüb[9] MT 35/42) at 38˚C.

[7] Corning Incorporated, Corning New York 14831, U.S.A
[8] ARC-Onderstepoort Veterinary Institute, P/bag X05 Onderstepoort, 0110, South Africa
[9] Minitüb, Abfüll-u, Labortecnik, GmbH and Co. KG 84184, Tiefenbach, Germany

3.3.2 Maturation of oocytes

Maturation of oocytes was done in batches of 100 - 110 COC's. Each batch was matured in a small petri dish (Corning 430588) containing 3 ml IVM medium (chapter 7.2) (31,68,69). The COC's were matured under mineral oil for 24 hours at 38°C in an atmosphere with 5% carbon dioxide in air and 100% humidity.

3.3.3 Fertilisation of oocytes

The semen that was used during IVF was from a Friesian bull that served as a control bull in the IVF laboratory of the Section of Reproduction, Onderstepoort. The semen met the minimum criteria set for frozen bovine semen (minimum 75% morphologically normal, minimum progressive motility of 40% and at least 7 x 10^6 progressive motile, morphologically normal sperm cells per 0.25ml French straw). Also, the semen was known to be negative for the presence of FMDV. The frozen semen was thawed in a waterbath at 35-37 °C for at least 20 seconds followed by swim-up separation as follows: Each half of the content of a 0.25 mℓ French straw was placed under FERT WASH medium (chapter 7.4) at 38 °C. The make-up of the Tyrode's Albumin–Lactate-Pyruvate (TALP) medium, used as the basis for preparation of FERT WASH is shown in Appendix A3 (chapter 7.3). The semen was left to swim-up for one hour at 38°C. The supernatant (±600 µℓ) was removed and centrifuged for 5 minutes at 300 X gravity. The resulting sperm pellet was resuspended in fertilisation medium (chapter 7.5 and 7.6) (70-72). After determination of sperm concentration with a Neubauer haemocytometer[10], oocytes were fertilised with 1x10^6 sperm per ml of the swim-up separated sperm. Approximately 50 000 swim-up separated sperm

[10] American Optical Corporation, Buffalo, New York 14215, U.S.A

cells, contained in 10 µl were added to 40 µl of fertilisation medium containing 10-15 COC's. The resulting 50 µl drops were co-incubated under mineral oil for 20 hours.

3.3.4 Culture of embryos

Sperm and cumulus cells were stripped from the presumptive zygotes, approximately 20 hours after the addition of sperm, by pipetting up and down several times in the 50 µl drops described above. During IVC, zygotes were cultured in 50 µl droplets of Synthetic Oviduct Fluid (SOF) (chapter 7.7), on granulosa cell derived monolayers. Granulosa cells that remained in the medium after IVM (when matured COC's were removed and submitted to IVF) were used in the preparation of the monolayers. The granulosa cells were cultured in 50 µℓ droplets of TCM 199-H supplemented with 10% steer serum and 25 µg/mℓ gentamycin under oil to create a monolayer (73). The number of zygotes cleaved to at least 2 cells was counted on day 3 of culture at the time of re-feeding the monolayers with SOF. On day 7 after fertilisation, all blastocysts were counted and graded according to IETS standards (12).

3.3.5 Addition of FMDV

Oocytes were co-incubated with FMDV, at a final concentration of $2x10^7$ $TCID_{50}$/ml, during IVM and IVF in different treatment groups as was described for the two separate experiments. No FMDV was added to the oocytes of the control groups. The Exotic Diseases Division of the Onderstepoort Veterinary Institute provided the FMDV used during the experiments. The same virus (SAT1, KNP 196/91/1), at the same concentration, was used for all the different treatment groups and for all experiments.

3.3.6 Treatment with MES

The acid that was used during experiment 2 was 2-[N-Morpholino]ethanesulfonic acid (MES, Sigma M2933). The MES was made up as MES Buffered Saline with pH 5.5 and pKa 6.1, prepared by adding 2.5 ml 1M MES and 10 ml 1.45M NaCl to 87.5 ml deionised water. MES, as used in the description of these experiments, refers to MES Buffered Saline.

Oocytes were treated with MES in the following way: One hundred oocytes/embryos were transferred with a 200 µl pipette to 1 ml of MES (200 µl of medium transferred to 1 ml of MES did not change the pH of the MES solution; pilot trial, unpublished results). The oocytes/embryos (contained in no more than 300 µl of MES) were then transferred (using a separate 500 µl pipette) to 3 ml FERT WASH in a small petri dish. The oocytes/embryos were in the MES for a minimum of 30 seconds and a maximum of 1 minute. The oocytes/embryos were washed five times through 5 ml aliquots of FERT WASH to get rid of MES. No more than 50 µl of wash fluid was transferred from one wash to the next and a new pipette tip was used for each of the steps.

3.3.7 Diagnostic tests for FMDV

The Exotic Diseases Division of the Onderstepoort Veterinary Institute routinely uses two diagnostic tests to diagnose and type viruses in the event of an FMDV outbreak. These are a PCR to detect viral genomic material and PKCC to isolate virus together with a subsequent ELISA to determine the serotype (3). These two tests were used in this study. It was decided to use both these tests, because the PCR could yield a positive result when viral RNA was detected, but this does not necessarily imply that the virus was still alive and capable of

infecting other cells. The PKCC was used to confirm that the virus, if any was detected with PCR, was still virulent and capable of causing cytopathic effects in PK cells.

3.3.7.1 The PCR technique as a diagnostic test for FMDV

A modified guanidinium thyocianate/silica method from Boom and co-workers (76) was used for the rapid extraction of viral RNA. Complementary DNA was prepared using a primer that recognises sequence at the 2A/B junction (5′ GAA GGG CCC AGG GTT GGA CTC 3′) described by Beck and Strohmaier (77).

Two different genes are targeted by the PCR. The universal primers target the polymerase gene, which confirms the presence of FMDV in a sample. A second primer targets the VP1 (ID) gene and the products of this primer can be used to determine the serotype of the virus (3). For this study, only the universal primers (5′ TGACCAAGAACAAAACCTTTG 3′ and 5′ CCACCCTGATGTTGATTGGC 3′) were used due to their higher sensitivity.

The standard operating procedure to extract viral RNA, synthesise cDNA, hot start PCR amplification and agarose gel electrophoresis of PCR products in the Exotic Diseases Division are given in Appendix B1 - B4 (chapter 8.1, 8.2, 8.3 and 8.4).

3.3.7.2 The primary pig kidney cell culture technique as a diagnostic test for FMDV

Pig kidney cells are highly susceptible to infection with FMDV. Monolayers of these cells show cytopathic effects when infected with FMDV, and thereby enable detection of infective FMDV (3). This technique makes use of standard cell culture techniques at the Exotic

Diseases Division of the Onderstepoort Veterinary Institute (3,62). Preparation of the primary PKCC is described in appendix B5 (chapter 8.5). Standard operating procedure for virus isolation with PKCC culture is given in Appendix B6 (chapter 8.6).

4 RESULTS

In Experiment (E1) FMDV could still be detected after washing of denuded zygotes or IVP embryos.

In experiment 2 (E2), FMDV could not be detected if denuded IVP zygotes were exposed to MES. FMDV could however still be detected if non-denuded structures were treated with MES.

4.1 Experiment 1: attempt to remove FMDV by washing

There was no significant difference between any of the replicates in each group and therefore total numbers of oocytes per group was used to test for differences between groups.

Group 1:

Cleavage, as assessed on day 3 of IVC was 62% (155/250) for E1G1. Fifty-five blastocysts were counted on day 7 after fertilisation. (blastocyst rate = 22%).

No FMDV could be detected in any of the embryos or in the IVC medium (Figure 4.1).

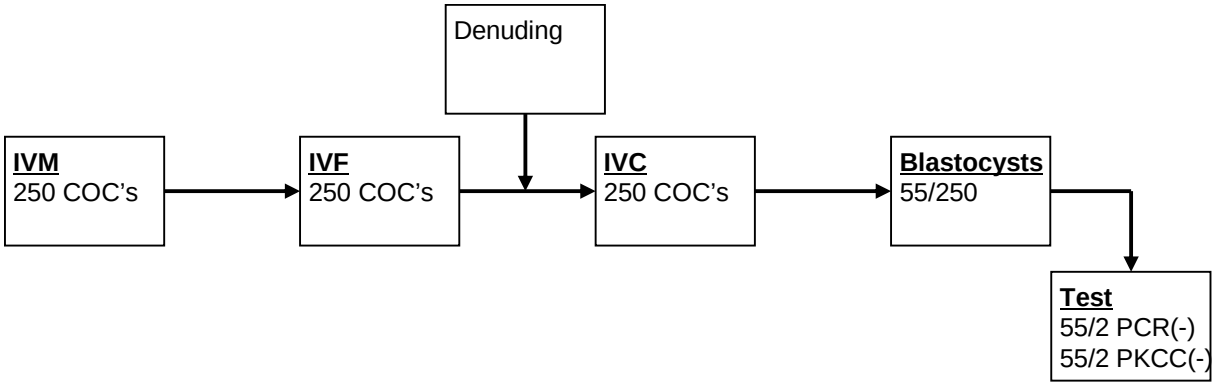

Figure 4.1: Experiment 1, Group 1, (E1G1). Flow-diagram for the results of the control group. Fifty-five blastocysts were cultured out of 250 COC's. No virus was added to this group and all blastocysts were negative for the presence of FMDV.

Group 2:

In E1G2, COC's were co-incubated with FMDV during IVM. FMDV was not added again during fertilisation and virus detection was attempted after denuding the zygotes.

The ten randomly selected COC's that were removed from this group after IVM induced cytopathic effect in PKCC within 24 hours and gave a positive result on PCR.

Similarly, the ten randomly selected COC's that were removed from this group after IVF induced cytopathic effect in PKCC within 24 hours and gave a positive result on PCR.

The denuded zygotes, as well as the cumulus cells yielded a positive result on both the cell culture and the PCR (Figure 4.2).

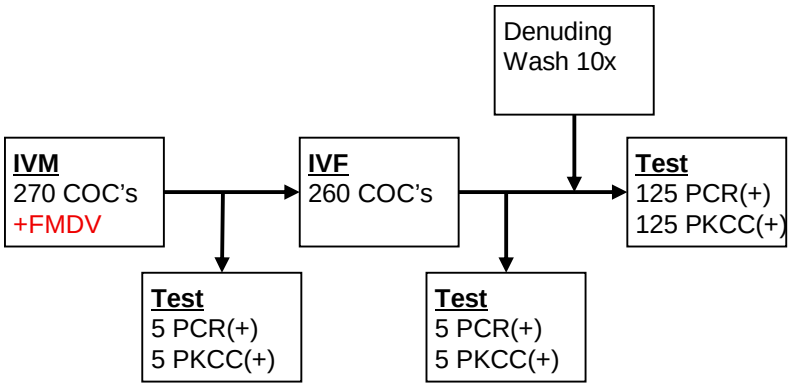

Figure 4.2: Experiment 1, Group 2, (E1G2). Flow diagram for the results. COC's were co-incubated with FMDV. Washing of denuded structures was done after IVF. FMDV was still present after denuding and washing.

Group 3:

In E1G3 COC's were co-incubated with FMDV during IVM. FMDV was not added again during IVF. The zygotes were submitted to IVC before detection of FMDV was attempted in the resulting embryos on day 7.

Cleavage, as assessed on day 3 of IVC was 58.8% (147/250) for this group. Forty-two blastocysts were counted on day 7 after fertilisation (blastocyst rate = 16.8%).

The ten randomly selected COC's that were removed from this group after IVM induced cytopathic effect in PKCC within 24 hours and gave a positive result on PCR.

Similarly, the ten, randomly selected zygotes that were removed from this group after IVF induced cytopathic effect in PKCC within 24 hours and gave a positive result on PCR.

The embryos that were produced at the end of IVC, washed ten times according to IETS standards, and submitted to virus detection using PKCC and PCR, yielded positive results on both the cell culture and the PCR (Figure 4.3).

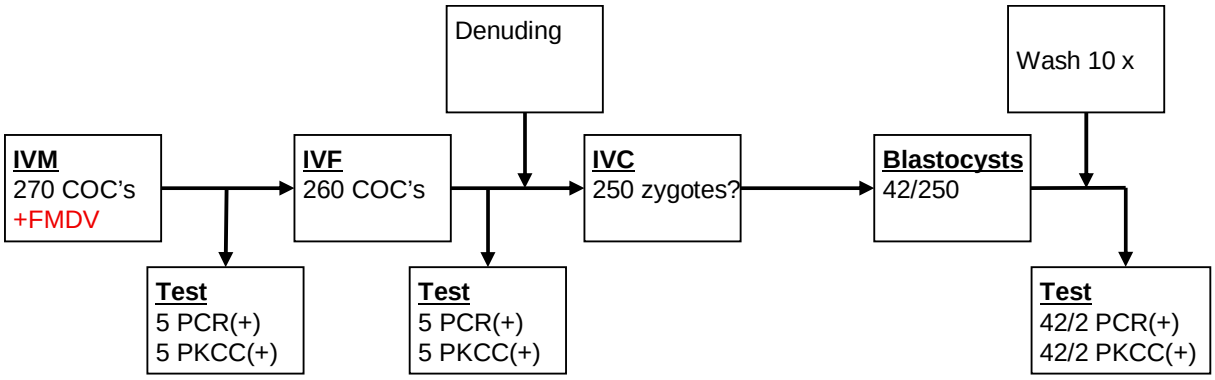

Figure 4.3: Experiment 1 Group 3 (E1G3). Flow diagram for the results. Forty-two blastocysts were created out of 270 COC's. FMDV was added during IVM. Blastocysts were washed after IVC. FMDV could still be detected in the embryos after washing.

Group 4:

In E1G4, COC's were co-incubated with FMDV during IVM. FMDV was added again during IVF. The zygotes were submitted to IVC before detection of FMDV was attempted in the resulting embryos.

Cleavage, as assessed on day 4 after fertilisation, was 52.8% (132/250) for this group. Forty-seven blastocysts were counted on day 7 after fertilisation (blastocyst rate = 18.8%).

The ten randomly selected COC's that were removed from this group after IVM induced cytopathic effect in PKCC within 24 hours and gave a positive result on PCR.

Similarly, the ten, randomly selected COC's that were removed from this group after IVF induced cytopathic effect in PKCC within 24 hours and gave a positive result on PCR.

The embryos that were produced at the end of IVC, washed ten times according to IETS standards, and submitted to virus detection using PKCC and PCR, yielded positive results on both the cell culture and the PCR (Figure 4.4).

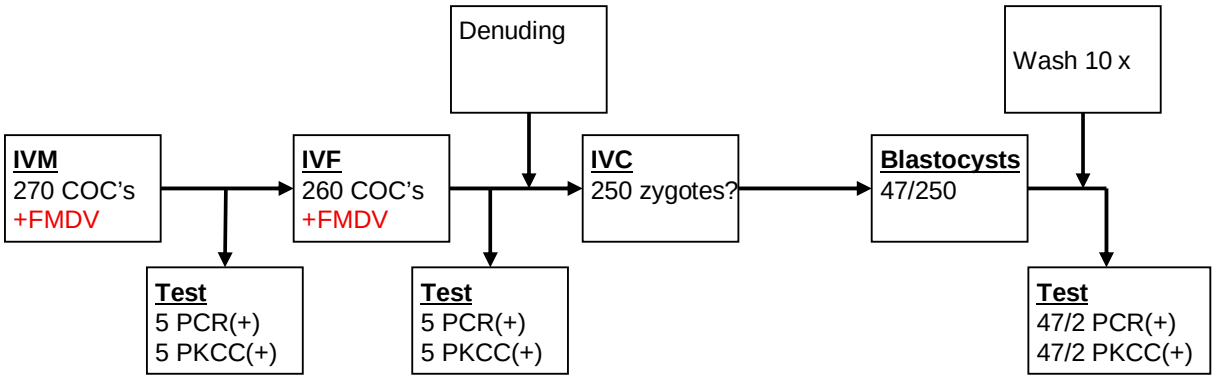

Figure 4.4: Experiment 1, Group 4, (E1G4). Flow diagram for the results. Forty-seven blastocysts were created out of 270 COC's. FMDV was added during IVM and IVF. Blastocysts were washed after IVC. FMDV could still be detected in the embryos after washing.

The results of experiment 1 are summarized in Table 4.1

Table 4.1: Summary of the results obtained for experiment 1.

	COC's[1] tested after IVM[2]	**COC's tested after IVF[3]**	**Denuded oocytes/emb. tested after IVF**	**Blastocysts tested**	**Cleavage rate**	**Blastocyst rate**
E1G1				PCR-[6] PKCC-[7]	62.0%	22.0%
E1G2	PCR+[4] PKCC+[5]	PCR+ PKCC+	PCR+ PKCC+			
E1G3	PCR+ PKCC+	PCR+ PKCC+		PCR+ PKCC+	58.0%	16.8%
E1G4	PCR+ PKCC+	PCR+ PKCC+		PCR+ PKCC+	52.8%	18.8%

[1]= Cumulus oocyte complex
[2]= *In vitro* maturation
[3]= *In vitro* fertilisation
[4]= Positive result for polymerase chain reaction (PCR)
[5]= Positive result for pig kidney cell culture (PKCC)
[6]= Negative result for polymerase chain reaction (PCR)
[7]= Negative result for pig kidney cell culture (PKCC)

A significantly lower cleavage was achieved in Group 4 when compared to the control group ($p<0.05$; Chi-square value = 3.9587). There was however no significant difference in blastocyst rate between the control group and any of the treatment groups.

4.2 Experiment 2: attempt to remove FMDV by acid exposure

On the day that the oocytes used in experiment 2 were aspirated, COC recovery was completed too late in the day for the oocytes to be transported to the Exotic Diseases Division. Therefore, the oocytes were left in the 5 ml round bevel cryogenic vials, each containing 100 oocytes in 3 ml IVM medium, in an incubator at 38˚C, with a 5% CO_2 atmosphere and 100% humidity overnight (12 hours). The COC's were transported to the Exotic Diseases Laboratory the next morning where the trials were conducted i.e., IVM was started after the 12 hour delay. IVM thus occurred over 36 hours in experiment 2.

There was not a statistically significant difference between any of the replicates in each group and therefore total numbers of oocytes per group was used to test for differences between groups.

Group 1:

No cytopathic effect was observed on the PKCC when the embryos or the culture medium in the control group (E2G1) were assessed for the presence of FMDV. No viral RNA could be detected with PCR in either the embryos or the culture medium.

On day 4 after fertilisation the cleavage rate was 45.3% (136 out of 300) and 25 blastocysts were produced on day 7 (blastocyst rate = 8.3%) (Figure 4.5).

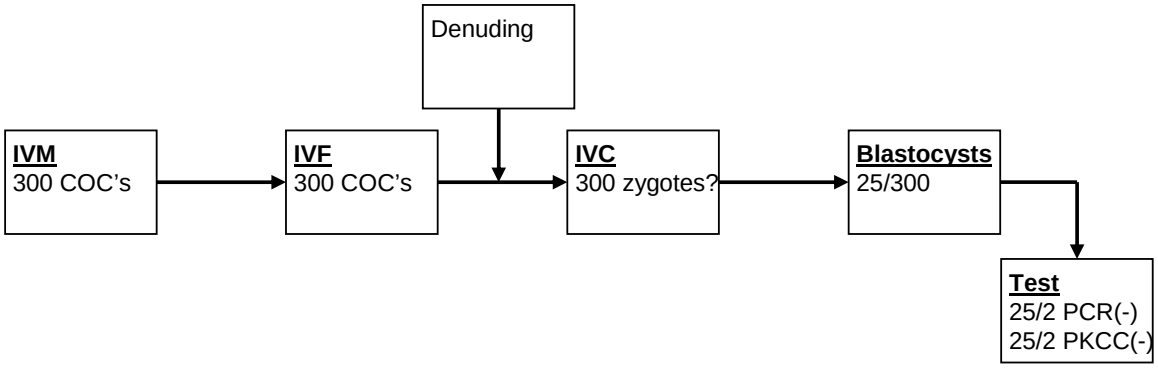

Figure 4.5: Experiment 2, Group 1, (E2G1). Flow diagram for the results. Twenty-five blastocysts were created out of 300 COC's placed in culture. No FMDV was added in the control group and none of the embryos tested positive for FMDV.

Group 2:

In E2G2 (Minimal MES treatment), COC's were co-incubated with FMDV during IVM. FMDV was again added during IVF. The zygotes were denuded after IVF and treated with MES. Virus detection was attempted after MES treatment.

The 5 oocytes, suspended in 1 ml PBS, that were removed after maturation and co-incubation with FMDV for 24 hours and put onto PKCC for virus detection, induced cytopathic effect in all of the PKCC's after incubation for 24 hours.

The PCR that was run on the 5 oocytes, suspended in 200 µl PBS, that were removed after maturation and co-incubation with FMDV for 24 hours was positive.

The 5 zygotes, suspended in 1 ml PBS, that were removed after IVF in the presence of FMDV, induced cytopathic effect in all of the PKCC's they were put onto.

The PCR run on the 5 zygotes, suspended in 200 µl PBS removed after IVF in the presence of FMDV, was positive.

No cytopathic effect was observed in the PKCC in any of the denuded zygotes in this group following treatment with MES. Similarly, no viral RNA could be found using PCR in any of the zygotes (Figure 4.6).

These zygotes were not cultured after IVF and consequently there are no cleavage and blastocyst rates for this group.

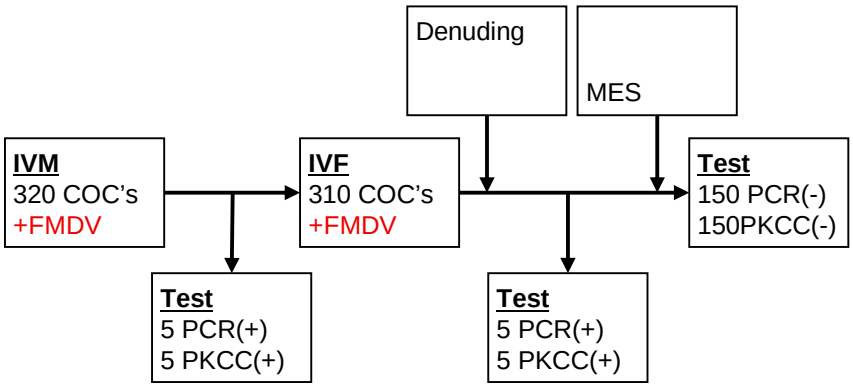

Figure 4.6: Experiment 2, Group 2, (E2G2; minimal MES treatment). Flow diagram for the results. FMDV was added during IVM and IVF. Denuded structures were treated with MES after IVF. None of the denuded structures was positive for FMDV after MES treatment.

Group 3:

In E2G3 (Minimal MES treatment), COC's were co-incubated with FMDV during IVM. FMDV was again added during IVF. The denuded zygotes were treated with MES after IVF. The zygotes were submitted to IVC in this group and virus detection was attempted in the resulting blastocysts.

The 5 oocytes, suspended in 1 ml PBS, that were removed after maturation and co-incubation with FMDV for 24 hours and put onto PKCC for virus detection, induced cytopathic effect in all of the PKCCs after incubation for 24 hours.

The PCR that was run on the 5 oocytes, suspended in 200 µl PBS, that were removed after maturation and co-incubation with FMDV for 24 hours was positive.

The 5 zygotes, suspended in 1 ml PBS, that were removed after IVF in the presence of FMDV, induced cytopathic effect in all of the PKCC's they were put onto.

The PCR run on the 5 zygotes, suspended in 200 µl PBS removed after IVF in the presence of FMDV, was positive.

No cytopathic effect was induced in the PKCC with any of the embryos on day 7 after fertilisation in this group. Similarly, no viral RNA could be found using PCR in any of the embryos.

On day 4 after fertilisation the cleavage rate was 43% (129 out of 300) and 31 blastocysts were produced on day 7 (blastocyst rate = 10.3%) (Figure 4.7).

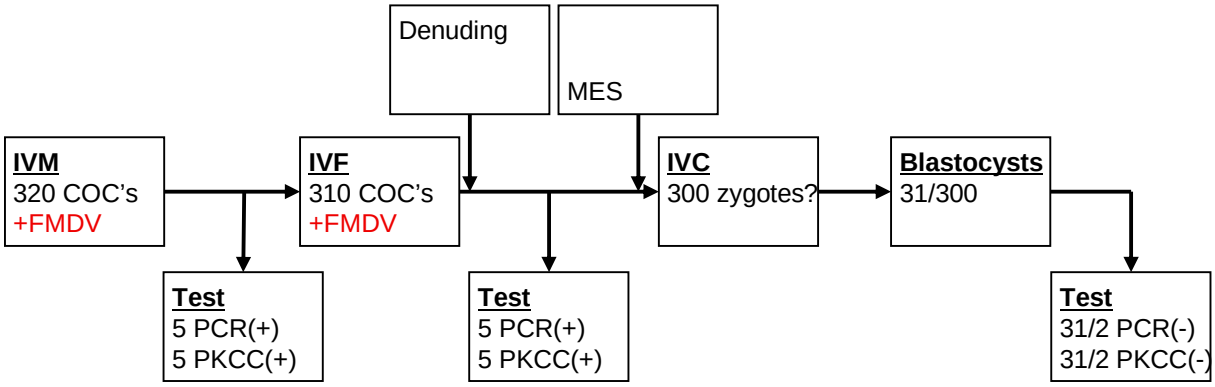

Figure 4.7: Experiment 2, Group 3, (E2G3; minimal MES treatment). Flow diagram for the results. Thirty-one blastocysts were created out of 320 COC's. FMDV was added during IVM and IVF. Denuded structures were treated with MES after IVC. None of the blastocysts tested positive for FMDV.

Group 4:

In E2G4, COC's were treated with MES after IVM and co-incubation with FMDV. Virus detection was attempted in the COC's after IVM and MES treatment. All of the PKCC's showed cytopathic effect and the PCR was also positive (Figure 4.8).

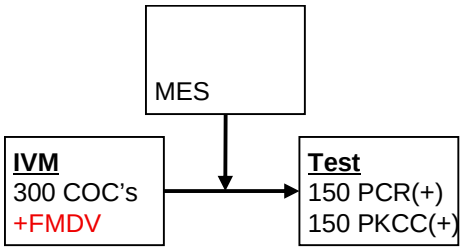

Figure 4.8: Experiment 2, Group 4, (E2G4). Flow diagram for the results. COC's were co-incubated with FMDV. MES treatment was done after IVM. FMDV could still be detected after MES treatment of the COC's.

Group 5:

In E2G5 (Maximal MES treatment), COC's were co-incubated with FMDV during IVM. FMDV was again added during IVF. Denuded zygotes were treated with MES after IVF and virus detection was attempted.

In this group none of the PKCC's showed cytopathic effect after 3 days of incubation. The PCR was negative (Figure 4.9).

The zygotes were not cultured after IVF and consequently there are no cleavage and blastocyst rates for this group.

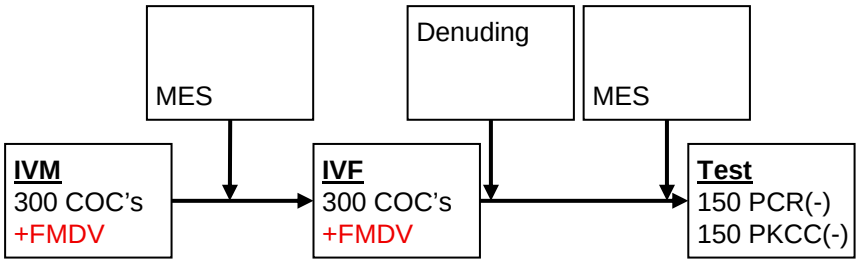

Figure 4.9: Experiment 2, Group 5, (E2G5; maximal MES treatment). Flow diagram for the results. FMDV was added during IVM and IVF. MES treatment was done after IVM in COC's and after IVF in denuded structures. None of the denuded structures tested positive for the presence of FMDV.

Group 6:

In E2G6 (Maximal MES treatment), COC's were co-incubated with FMDV during IVM. FMDV was again added during IVF. Denuded zygotes were treated with MES before submission to IVC. Virus detection was attempted in the resulting blastocysts.

None of the PKCC's showed cytopathic effect after 3 days of incubation.

The PCR was negative.

On day 4 after fertilisation, the cleavage rate was 44% (132 out of 300) and 28 blastocysts were produced on day 7 after fertilisation (blastocyst rate = 9.3%) (Figure 4.10).

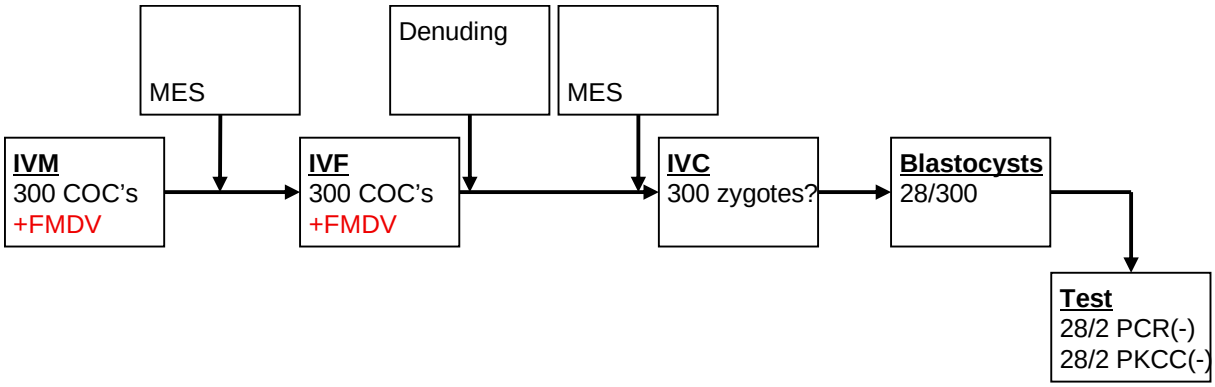

Figure 4.10: Experiment 2, Group 6, (E2G6; maximal MES treatment). Flow diagram for the results. Twenty-eight blastocysts were created out of 300 COC's. FMDV was added during IVM and IVF. MES treatment was done after IVM and on denuded structures after IVF. None of the blastocysts tested positive for the presence of FMDV.

The results for experiment 2 are summarized in Table 4.2.

Table 4.2: Summary of results obtained for experiment 2

	COC's[1] tested after IVM[2] (before MES[3] treatment)	**COC's tested after IVM (after MES treatment)**	**Denuded oocytes/emb. tested after IVF[4] (before MES treatment)**	**Denuded oocytes/emb. tested after IVF (after MES treatment)**	**Blastocysts tested**	**Cleavage rate**	**Blastocyst rate**
E2G1					PCR-[5] PKCC-[6]	45.3%	8.3%
E2G2 (minimal MES treatment)	PCR+[7] PKCC+[8]		PCR+ PKCC+	PCR- PKCC-			
E2G3 (minimal MES treatment)	PCR+ PKCC+		PCR+ PKCC+		PCR- PKCC-	43%	10.3%
E2G4		PCR+ PKCC+					
E2G5 (maximal MES treatment)				PCR- PKCC-			
E2G6 (maximal MES treatment)					PCR- PKCC-	44%	9.3%

[1]= Cumulus oocyte complex
[2]= *In vitro* maturation
[3]= 2-[N-Morpholino]ethanesulfonic acid
[4]= *In vitro* fertilisation
[5]= Negative result for the polymerase chain reaction (PCR)
[6]= Negative result for the pig kidney cell culture (PKCC)
[7]= Positive result for the polymerase chain reaction (PCR)
[8]= Positive result for the pig kidney cell culture (PKCC)

5 DISCUSSION

Two methods were employed in order to render IVP embryos free from FMDV. In experiment 1 routine washing procedures as described by IETS (12) was used and in experiment 2 treatments with an organic buffer solution was performed.

FMDV could not be removed by washing. It is not clear whether FMDV infects cumulus cells, if it is simply associated with the cumulus cells on the outside of the cell membrane, if it is inside the pores of the ZP, or if it penetrates the ZP.

The possibility of cumulus cell infection, or at least attachment of the virus to cumulus cells, seems most likely, because, subjectively assessed with 80X magnification; cytopathic effect was evident in the cumulus cells after co incubation with FMDV. The cumulus cells in the groups co-incubated with FMDV were expanded, but not uniformly so. Some cells clumped together and were also darker than the surrounding cells. None of these changes were noted in the control groups.

It seems unlikely that FMDV penetrates the ZP and is protected from the effects of washing by residing inside the ZP. If this were the case, one would expect impaired development of embryos (assuming FMDV will infect the embryos), because attachment of FMDV to embryonic cells can be damaging to them (1). Development of embryos does not seem to be impaired in these experiments, because cleavage and blastocyst production rates were not significantly different from the control groups (Figure 3.3 and Figure 3.4). Also, no morphological differences were noted between the embryos in the FMDV-treated groups and the control groups.

It is possible that FMDV can enter the pores in the ZP of IVP embryos and be protected from the effects of washing inside the pores. In contrast, FMDV does not seem to associate in the same way to *in vivo*-derived embryos and, consequently, can be cleared by washing according to IETS standards (12,59,60). These results are in accordance with previous results where IVP embryos were exposed to FMDV seven days after IVF (49). Differences between the ZP of follicular oocytes, *in vivo* -and *in vitro* -embryos have been documented (38). It is also possible that differences in the ZP of follicular oocytes and *in vivo* -derived embryos allow FMDV to adhere more firmly to the ZP of follicular oocytes and washing alone is ineffective in clearing the ZP from the virus. It is however clear that greater care should be taken when dealing with IVP embryos, because embryo-pathogen interactions are not necessarily the same as for *in vivo*-produced embryos. Similar associations have been reported for IVP embryos and BHV-1 (50,51) and BVDV (52).

When subjectively assessed, cytopathic effects induced in PKCC were less (fewer cells per tube showed cytopathic effect) for the denuded zygotes than for the 10 COC's removed after maturation and fertilisation in E1G2. This observation could be due to a lower concentration of FMDV associated with denuded zygotes when compared to cumulus cell-associated structures. The reasons for this are unclear, but are probably related to the way in which the virus associates with the oocytes, cumulus cells and embryos. This conforms to IETS standards, which state that the ZP must be intact and free from adherent material before washing is attempted. More research is needed to elucidate conclusively how the FMDV associates with cumulus cells and ZP respectively. It does however seem that washing denuded IVP zygotes according to IETS standards significantly decreases the amount of virus associated with them, but it does not render them completely free of infective FMDV. In a

consecutive trial it would be recommended to do titrations and calculate the concentration of FMDV associated with the different structures in order to assess the extent to which virus can be reduced by washing.

These experiments were designed to reflect natural exposure of cows and bulls to FMDV and the subsequent use of such material in an *in vitro* embryo production system. In a previous study, IVP embryos were exposed to and co-incubated with FMDV 7 days after IVF, and washing was done between 1 and 4 hours later (49). Although it was not possible to completely remove all the virus, these studies did not reveal information on the association of FMDV with COC's and did not investigate the effects of infected semen in an *in vitro* embryo production system. It is clearly evident from these experiments that the association of FMDV with COC's, zygotes and embryos during *in vitro* embryo production are very different from the association of the virus with *in vivo* embryos when these results are compared to the results where washing was done on *in vivo* embryos (59,60).

The media used for the IVP of bovine embryos did not appear to affect the viability of the FMDV, and the culture conditions for the embryos appeared to be supportive to the survival of FMDV. Live virus, capable of inducing cytopathic effects in PKCC monolayers, was isolated in COC's that were removed after IVM and after IVF in the Groups E1G2-4 and E2G2-3. Also, live virus was isolated associated with denuded structures at the end of IVC in Groups E1G3 and E1G4.

Further work is needed on the association of FMDV with bovine semen. It would be interesting to test if cows inseminated with semen from a viraemic bull would yield embryos positive for FMDV and if so, if it could be removed by embryo washing. It is known that

FMDV is excreted in bovine semen (74), but it is not known if the virus can be carried into the embryo along with a sperm head. Most of the pathogens, if not all, reported in the semen of livestock are found free in the seminal plasma rather than within or attached to the spermatozoa (12,75). Sperm tracts are usually single and tend to have a shallow, curving trajectory through the ZP. These tracts seem to close soon after fertilisation. It is virtually impossible for viruses, which are non-motile, to pass through the ZP via a sperm tract, because the tracts close so quickly (12).

Treatment of COC's with MES for between 30 and 60 seconds was not effective for complete removal of the virus (E2G4). This might be because the virus gets trapped and protected from MES exposure in gaps between the cumulus cells or the virus has become internalised by the cumulus cells and thus protected from the acid environment. If the virus was trapped and protected in gaps between the cumulus cells, longer exposures to MES might be effective in destroying the virus by allowing more time for the MES to penetrate in between the cumulus cells. It is however unknown how longer exposures to MES would affect the cumulus cells or oocytes. More research is needed to accurately assess the effect of MES on these structures. It is also possible that differences in the ZP structure of COC's are responsible for this result, because FMDV might be protected from the acid environment in pores of the ZP. For the same reasons as noted earlier, the most likely reason is that FMDV infects cumulus cells and is protected inside the cells during acid treatment. Cumulus cell damage as described earlier was evident in the groups that were exposed to FMDV when subjectively evaluated with a stereomicroscope with 80X magnification. This cytopathic effect is likely to be because of attachment of FMDV or infection with FMDV. Cumulus cells in groups that were not exposed to FMDV did not show the same changes. In a follow - up trial, samples of cumulus cells should be exposed to FMDV and treated with MES afterwards. If cumulus cells test

positive for FMDV after MES treatment this would suggest that the virus is inside of the cumulus cells because MES treatment would have destroyed all virus on and outside of the cumulus cell membrane. Additional study is needed to investigate the association of FMDV with COC's and in particular cumulus cells.

Treatment of denuded zygotes with MES was effective to get rid of infective FMDV on them. None of the samples of denuded zygotes, in any of the different treatment groups, induced cytopathic effect in PKCC indicating that infective virus was not present. Acidic solutions have been shown to disintegrate FMDV into its subunits (1). Although this liberates infectious RNA, it is usually rapidly inactivated by environmental ribonucleases (1,53). No viral RNA could be detected when PCR was used for virus detection indicating destruction of at least the polymerase gene of FMDV, which is the target for the PCR (5). This result also suggests that FMDV does not penetrate the ZP, and that the association with the ZP of denuded zygotes was such that treatment with acid destroyed it. If FMDV enters the pores of the ZP where it can be protected from the effects of washing, MES must also have penetrated the pores to at least the same depth as the virus. If the virus penetrated the ZP, one would expect positive PKCC and PCR results, because the virus would have been protected from the effects of MES. The ZP is however destroyed for both the PKCC and PCR procedures and if infective virus was within the embryo proper these tests should have detected it.

The concentration of FMDV that was used during these trials was very high. It was at least twice as high as the highest titre recorded for urogenital secretions in cattle (1,4). It is unlikely that such high concentration of virus would occur naturally in follicular fluid or semen, and that the virus could be introduced into the *in vitro* embryo production system in such high quantities. It is therefore reasonable to accept that bovine embryos matured and

fertilised while co-incubated with FMDV can be rendered free from infectious virus by treating them with MES. Where the possibility exists that FMDV could be introduced into an *in vitro* system, it should be recommended that denuded structures be treated with MES after IVF and embryos be washed according to IETS standards after IVC.

MES treatment during these experiments was done before embryos/oocytes were washed. This was done, because it was expected that if embryos/oocytes were washed first and then treated with MES the washing procedure would have lowered the virus concentration before MES treatment was done. These experiments were designed to reflect the worst-case scenario where very high levels of FMDV are present before treatment with MES, and to subsequently get rid of the virus again. For the same reason MES treatment was not done in the middle of ten washes (trypsin treatment is usually done after the 5th wash when washing embryos according to the IETS protocol) to prevent reduction of FMDV levels before exposure to MES. When there is a risk that FMDV could be present in the *in vitro* system, it should be recommended that denuded structures be washed before MES treatment to lower the amount of virus present as an additional safeguard.

It appears that treatment of COC's or denuded zygotes with MES is not harmful to the oocytes or embryos and that treatment with MES does not alter production of IVP embryos. The blastocyst production rate however was not optimal and further investigation is needed to determine the effect of MES treatment during the production of *in vitro* embryos. It might well be that treatment with MES is in fact detrimental to embryos in an *in vitro* system, but that the blastocyst numbers were too small during these experiments and the power of the statistical test that was used might have been inadequate to detect small differences between the control and treatment groups. During a follow up trial, oocytes/embryos should be

exposed to MES during *in vitro* production of embryos under the controlled environment of an *in vitro* embryo production laboratory to accurately measure the effects of MES on these structures.

It appears that FMDV is not overtly harmful to oocytes/embryos exposed to the virus at concentrations used during these trials. This can be concluded, because there is no statistical difference in cleavage rate and blastocyst rate between the control groups and the groups that were exposed to FMDV. However, blastocyst numbers were small and the power of the test used might have been inadequate to demonstrate small differences. It is possible that differences between control and treatment groups will become apparent when larger blastocyst numbers are analysed. As described earlier, it was noted that the cumulus cells were not as healthy in the groups that were co-incubated with FMDV as compared to the control groups when examined under a stereomicroscope at 80X magnification. None of these changes were noted in the control groups. It is not known if these changes would affect the pregnancy rates achieved by such embryos. None of these embryos have been transferred into recipients to investigate pregnancy rates. Also, none of these embryos were cultured for longer than seven days after fertilisation to investigate hatching rates achieved by embryos that were co-incubated with FMDV. Further work is needed to investigate the hatching rates and ultimately pregnancy rates in oocytes/embryos exposed to FMDV during *in vitro* embryo production.

Few of the published FMDV PCR primer pairs fulfil both requirements for good primers, namely the detection and the characterization of the virion. The identification of a primer pair, which amplifies all seven serological types and has a wide range of recognition for the many subtypes, is critical for the identification of field strains of FMDV in the case of an

outbreak. The products of such a primer pair should be amenable to sequencing to allow characterization of the virus. Bastos (1998) described a primer pair that is capable of detecting FMDV within 6h of receiving a sample and allows for accurate genetic characterisation on the basis of nucleotide sequencing within 48 h of receiving a positive sample. The primers described by Bastos (1998) resulted in 100% detection of all SAT-types tested and fulfil the requirements of FMDV detection and characterization. Furthermore, the virus used during these experiments was known to be detected by the primers described by Bastos.

The overall blastocyst production rate in this study is lower than the average for the *in vitro* laboratory at Onderstepoort (average blastocyst production rate for the two experiments = 14.25% compared to laboratory average of about 30%) The blastocyst production rate in experiment 1 is higher (19.2%) than that in experiment 2 (9.3%). However, the blastocyst production rate between the different treatment groups in each experiment did not differ significantly. A significantly lower cleavage rate was recorded for Group E1G4 (in E1G4 FMDV was added to the system during IVM and IVF. Washing of embryos was done on day 7) when compared to the control group (E1G1). We do not think that this is a biologically meaningful difference since the blastocyst rate in E1G4 did not differ significantly from that in E1G1. These experiments were performed at the Exotic Diseases Division of the Onderstepoort Veterinary Institute and not in the *in vitro* laboratory at the Faculty of Veterinary Science where IVP embryos are usually cultured. The Exotic Diseases Division is not designed for the production of embryos *in vitro* and conditions during the completion of these trials were not optimal. Oocytes/embryos were often outside of the incubators longer than necessary, because the incubators were not located close to the

working bench area. Also, the temperature regulation in the Exotic Diseases Division was not optimal for the production of embryos *in vitro*.

The poorer cleavage and blastocyst production rates during experiment 2 when compared to experiment 1 could be ascribed to less that optimal handling of the COC's during transfer to the Exotic Diseases Division laboratory. On the day that the ovaries were collected from the abattoir, aspiration of the COC's was completed too late in the day to transfer them from the *in vitro* laboratory at the Faculty of Veterinary Science to the Exotic Diseases Division as mentioned earlier. Maturation of COC's in experiment 2 occurred over a period of 36 hours instead of 24 hours because of this delay

6 SUMMARY AND CONCLUSION

No other references could be found in the literature where IVP embryos, exposed to FMDV were rendered free of the virus. To the best of my knowledge this is the first report that describes the association of FMDV with IVP embryos during IVM and IVF. Also, this is the first description of a treatment protocol where IVP embryos can be cleared of infective FMDV. Washing embryos in accordance with IETS regulations was ineffective in clearing FMDV from IVP embryos when IVM and IVF were done in the presence of FMDV; however, treatment with acid as described during these experiments was effective for achieving FMDV - free IVP embryos.

This work might enable the production of IVP embryos in countries where FMDV is endemic, permitting the subsequent movement of the embryos to other countries. Also, it might serve as a way of preserving genetic material from animals during an outbreak of the disease, in countries where FMD is not endemic, through the production of disease-free embryos.

This work might also be of use to the wildlife industry in South Africa. If a protocol was developed for the *in vitro* production of game animal embryos, notably buffalo, treatment with MES could enable the production of embryos free of FMDV. This would facilitate movement of embryos from areas in South Africa where FMDV is endemic.

7 APPENDIX A (MEDIA)

7.1 Appendix A1

Phosphate Buffered Saline (PBS)

Na_2HPO_4 (Sigma, S5011)	5.57 g
KH_2PO_4 (Sigma, P5655)	1 g
NaCl (Sigma, S7653)	40 g
KCl (Sigma, P5405)	1 g
H_2O (Sigma, W1503)	5 l
Osmolarity	280-300mOsM/kg
pH	7.4-7.6

7.2 Appendix A2

IVM medium

Stock medium

H_2O (Sigma, W1503)	1000 ml
TCM199-H (Sigma H2520)	15 g
$NaHCO_3$ (Sigma, S5761)	2.2 g
pH	7.4
Osmolarity	290-310

Working solution

Stock medium	50 ml
Steer serum	2.5 ml
Gentamycin (Sigma, G1264)	25 µl
Mercaptoethylamine (Sigma, M5600)	500 µl

7.3 Appendix A3

Sperm TALP (Stock media)

H_2O (Sigma, W1503)	1000 ml
NaCI (Sigma, S7653)	6.66 g
KCI (Sigma, P9541)	0.238 g
$NaHCO_3$ (SigmaS5761)	2.090 g
$NaHPO_4(1H_2O)$ (Sigma, S9638)	0.055 g
HEPES (Sigma, M9136)	2.4 g
$CaCI_2$ (Sigma, C7902)	0.294 g
$MgCI_2$ (Sigma, M2393)	0.1 g
Phenol red (Sigma, P5530)	1 ml
pH	7.4
Osmolarity	275-280

7.4 Appendix A4

FERT WASH medium

Sperm TALP (Stock medium)	50 ml
Gentamycin (Sigma G1264)	25 µl of a 50 mg/ml solution
Bovine serum albumin-Fatty acid free (Sigma A8806)	0.3 g
Pyruvate (Sigma, P5280)	0.0028 g
Lactate (Sigma, L4263)	67 µl
pH	7.4

7.5 Appendix A5

PHE

Solution A

H_2O (Sigma, W1503)	33 ml
NaCl (Sigma, S7653)	0.3 g

Solution B

Solution A	20 ml
Penicillamine (Sigma, P4875)	0.003 g
Hypotaurine (Sigma, H1384)	0.00109 g

Solution C

H_2O (Sigma, W1503)	50 ml
Metabisulphate (Sigma, S9000)	0.05 g
Lactate (Sigma, L4263)	110 µl
pH	4
Epinephrine (Sigma, E4250)	0.00228 g

Working Solution

Solution A	6 ml
Solution B	10 ml
Solution C	4 ml

7.6 Appendix A6

Fertilisation medium

FERT WASH	50 ml
Heparin (Sigma, H9399)	0.0015 g
PHE	2 ml
pH	7.8

7.7 Appendix A7

SOF medium

Stock medium:

H_2O(Sigma, W1503)	100 ml
NaCI (Sigma, S7653)	0.6294 g
KCI (Sigma, P9541)	0.05338 g
KH_2PO_4 (Sigma, P5655)	0.0162 g
$CaCl_2$ (Sigma, C7902)	0.0251 g
$NaHCO_3$ (Sigma, S5761)	0.2106 g
$MgCI_2$ (Sigma, M2393)	0.00996 g

Medium:

Stock medium	9.5 ml
Lactate (Sigma, L4263)	2.25 µl
Pyruvate (Sigma, P5280)	0.00033 g
Glutamine (Sigma, G1517)	0.00146 g
Gentamicin (Sigma, G1264)	5 µl
Steer serum	500 µl
MEM Non-essential amino acid solution (Sigma, B6766)	100 µl
BME Amino acids solution (Sigma, M7145)	200 µl

All the *in vitro* media described above were filtered through a 0.2 µl filter (MSI, Westboro, MA).

For all the media described above, the pH was adjusted using HCl (Merck, BD190686W) and NaOH (Merck, BD, 191396T).

8 APPENDIX B (SOP)

8.1 Appendix B1

STANDARD OPERATING PROCEDURE FOR RNA EXTRACTIONS IN THE EXOTIC DISEASES LABOROTORY

RNA EXTRACTION - MODIFIED GUANIDINIUM-BASED NUCLEIC ACID EXTRACTION METHOD (76).

The working protocol describes the extraction of RNA from clinical samples and tissue culture samples.

1. MATERIALS AND EQUIPMENT

1.1 Materials

- Silica (See 3.1 for preparation)
- Lysis buffer (see 3.2 for preparation)
- Wash buffer (see 3.3 for preparation)
- 10 M NaOH (Merck)
- 70% EtOH (Merck)
- Acetone (Merck)
- 1 x TE (see 3.4 for preparation)
- RNasin (Promega)

1.2 Equipment

- 100 – 1000 µl pipette
- 20 – 200 µl pipette
- Filter Pipette tips
- 1,5 ml Eppendorf tubes
- 15 ml sterile Conical Centrifuge tubes (Nunc)
- Heidolph Vortex
- Bench top micro 12-24 Hettich centrifuge
- Thermolyne heating block
- Surgical gloves
- Labaire Fume hood

2. METHOD

2.1 Precaution

- Clean area before starting the work
- Work with gloves
- Perform Step 1 in a laminar flow cabinet

  Perform Steps 2-7 in a fume hood
- Check expiry date of all chemicals

2.2 Method

Volumes have been calculated for the extraction of nucleic acids from 6 samples. For variations on the number of samples see end of section.

1. Remove an aliquot of 200 µl of the sample with a P1000 micropipette (use filter tips) and transfer to a 1.5 ml Eppendorf tube.

2. Transfer 6 ml of L6 Lysis buffer to a sterile 15 ml blue cap tube. Vortex an aliquot of silica (see 3.1) (until completely in solution) and add 240 µl of the resuspended silica to the lysis buffer in the clear blue cap tube. Cap and vortex the tube. **NB:** Addition of silica mix (containing HCl) to lysis buffer MUST be performed in the fume hood due to the formation of cyanide gas.

3. Add 940 µl of L6/silica mix to each of the samples using a clean filter tip each time. Vortex the mixture and allow to stand at room temperature (RT) for 5 min. Invert the tubes at intervals during this incubation period while still working in fume hood.

4. Vortex the tubes (5s) and centrifuge (15s).

5. Remove the supernatant (pour off) and dispose of it in the 10M NaOH waste bottle. The nucleic acids are now bound to the silica pellet.

6. Add 900 µl of L2 wash buffer to the pellet (fume hood) and vortex each tube until the silica is completely in suspension. Centrifuge for 15 s to pellet the silica.

7. Set the P1000 micropipette on 1 ml and carefully remove the supernatant. Dispose of it in the 10M NaOH waste bottle.

8. Add 800 µl of 70% ethanol to the pellet, vortex and centrifuge. Pour off the supernatant.

9. Repeat step 8 with 700 µl acetone. Dry the silica pellets by placing the tubes (lids open) in a heating block preset to 56°C. Incubate for 10-20 minutes (until the pellets are completely dry).

10. Make up a 1xTE/RNasin mix as follows: 180 µl 1xTE + 1µl RNasin (40U/µl; Promega) for 6 reactions; 240 µl for 8 reactions and 300 µl for 10 reactions. Add 30 µl of the mixture to each tube (filter tip) and vortex.

11. Incubate the tubes for 2 min at 56°C. Centrifuge for 5-10 minutes and transfer the supernatant containing the eluted nucleic acids to a new, clean, RNase-free 1.5 ml eppendorph tube. Label the tubes.

12. Should any carry-over of silica occur when removing the supernatant (step 11), then repeat the 5-10 min centrifugation step and transfer silica-free supernatant to a new clean tube. Trace amounts of silica can interfere with subsequent enzymatic reactions and should be removed completely.

13. Immediately proceed with the cDNA synthesis or alternatively store the RNA at -70°C until needed.

No of	L6 lysis buffer	Silica

samples		
6	6 ml	240 µl
7	7 ml	280 µl
8	8 ml	320 µl
9	9 ml	360 µl
10	10 ml	400 µl

3. STOCK SOLUTIONS

3.1 Preparation of size fractionated silica

- Perform all steps in treated glass and wear gloves.
- Suspend 6 g of silicon dioxide 99% (Sigma) in a 50 ml volume of sterile ddH_2O in a 50 ml glass cylinder and allow to sediment for 24 hours at room temperature.
- Remove 43 ml of the supernatant portion by suction with a pipette-aid.
- Add sterile ddH_2O to the measuring cylinder so that a final volume of 50 ml is obtained.
- Resuspend the silica pellet by vigorous shaking.
- Allow a second sedimentation for 5 hours at room temperature.
- Remove 44 ml of the supernatant and add 60 µl of HCl (32% wt/vol) (Merck) in order to adjust to pH 2.
- Prepare 400 µl aliquots in 1.5 ml Eppendorf tubes and store in the dark.

3.2 L6 lysis buffer

Dissolve 60 g of Guanidine Thiocyanate (GuSCN- Promega) in 50 ml of 0.1M Tris-HCL (pH 6.4). Add 11 ml of 0.2M EDTA solution and 1.3 g of Triton X-100 (Roche)

Place at 50°C until completely in solution.

Wrap in foil and store at room temperature.

3.3 L2 wash buffer

Dissolve 60 g GuSCN (Promega) in 50 ml of 0.1M Tris-HCL (pH 6.4) by placing at 50°C.

Wrap in foil and store at room temperature.

3.4 1 x TE

1 ml - 1M Tris-HCl (pH 7.4)

1 ml - 1M EDTA (pH8.0)

Make up to 100 ml with ddH20

Autoclave

8.2 Appendix B2

STANDARD OPERATING PROCEDURE FOR cDNA SYNTHESIS IN THE EXOTIC DISEASES LABOROTORY

cDNA SYNTHESIS USING AMV-REVERSE TRANSCRIPTASE (77).

The working protocol describes the synthesis of cDNA from the isolated RNA to be used in PCR

1. MATERIALS AND EQUIPMENT

1.1 Materials

- 5 x AMV-RT buffer (Promega)
- AMV-RT enzyme (Promega)
- 2.5 mM dNTP (Roche) (see 3.1 for preparation)
- W-DA primer (see 3.3 for seqeuncing)
- Dimethylsulfoxide (DMSO) (MERCK)
- Random hexanucleotides (Roche)
- Rnase inhibitor (Promega)
- 70% Ethanol
- 1 x TE (see 3.2 for preparation)
- Surgical gloves

1.2 Equipment

- 5 µl – 40 µl pipette
- 0.5 µl – 10 µl pipette
- Pipette tips
- 0.5 ml Eppendorf tubes
- Heidolph Vortex
- Bench top micro 12-24 Hettich centrifuge
- Thermolyne Heating block
- Water bath
- Liquid nitrogen

2. METHOD

2.1 Precaution

- Clean area before starting the work with 70% ethanol
- Work with gloves

2.2 Preparation of cDNA

- Remove the 5 x AMV-RT buffer, 2.5mM dNTPs, random hexanucleotides and W-DA (10 pmol/µl) from the freezer and allow thawing. Make up a cDNA master mix (MM) according to the number of samples to be reverse transcribed in the following manner:

6x MM	8x MM	10x MM	Reagents

12 µl	16 µl	20 µl	5x AMV-RT buffer
4.8 µl	6.4 µl	8 µl	2.5mM dNTP
3 µl	4 µl	5 µl	W-DA primer (10pmol/µl)
3 µl	4 µl	5 µl	DMSO
1.5 µl	2 µl	2.5 µl	Random hexanucleotides (200pmol/µl)
1.5 µl	2 µl	2.5 µl	Diluted RNase inhibitor
1.5 µl	2 µl	2.5 µl	dH_20

- Make a dilution of RNase Inhibitor (Promega enzyme) as follows (depending on sample number) and place on ice:

2.3 Add 1 µl of RNasin (40U/µl) enzyme to

6.5 µl of 1xTE	(6 x MM)
9 µl of 1xTE	(8 x MM)
11.5 µl of 1xTE	(10 x MM)

- Centrifuge the master mix. Set the pipette to 4.5 µl and mix by pipetting the mixture well before aliquoting to 0.5 ml RNAse free tubes.

- Aliquot 4.5 µl of the cDNA mix to six 0.5 ml tubes. Label the tubes (isolate name, cDNA synthesis number and date) and place on ice.

- Add 4.5 µl of the diluted RNA extract to the correspondingly labelled 0.5 ml tube.

- Centrifuge and incubate at 70°C for 3 minutes in a water bath

- Place the samples on ice for 3 minutes. Whilst the tubes are on ice add AMV-RT (10U/µl; Promega) to the remaining diluted RNase inhibitor solution as follows: 6 µl AMV-RT (6 reactions); 8 µl AMV-RT (8 reactions), etc. Place on ice and mix thoroughly before aliquoting to each of the tubes.

- Aliquot 1.5 µl of the enzyme/RNase inhibitor mix to each of the cDNA tubes (immediately return the tubes to ice after adding the enzyme, and use a new tip each time).

- Centrifuge tubes briefly and incubate at 42°C for 1 hour.

- Inactivate the enzyme by placing at 80°C for 1 minute and store at 4°C.

3. STOCK SOLUTIONS

3.1 2.5 mM dNTP

25 µl dATP (Roche-PCR grade)

25 µl dCTP (Roche-PCR grade)

25 µl dGTP (Roche-PCR grade)

25 µl dTTP (Roche-PCR grade)

900 µl H20

3.2 1 x TE

1 ml - 1M Tris-HCl (pH 7,4)

1 ml - 1M EDTA (pH8,0)

Make up to 100 ml with ddH20

Autoclave

3.3 Primer

Published primer by Bastos (1998). Make a 10p/M dilution with 1x TE buffer

8.3 Appendix B3

STANDARD OPERATING PROCEDURE FOR HOT START PCR AMPLIFICATION IN THE EXOTIC DISEASES LABOROTORY

HOT START PCR AMPLIFICATION(5,77)

The working protocol describes the genomic amplification of a targeted region of the FMD viral genome.

1. MATERIALS AND EQUIPMENT

1.1 Materials

- 10x buffer (Roche)
- 2 mM dNTP's
- Sense primer
- Antisense primer
- Roche taq
- Surgical gloves
- Sterile mineral oil
- Sterile ddH_2O

1.2 Equipment

- Micro-pipette
- Micro-pipette filter tips

- 0.5 ml thin walled PCR tubes (Hybaid)
- PCR machine (Hybaid Omnigene)
- Bench top micro 12-24 Hettich centrifuge
- Labotec Bio-Flow Laminar flow

2. METHOD

2.1 Precaution

- Clean area before starting the work with 70% Ethanol
- Switch the UV light on 20 minutes before preparing the master mix
- Work with gloves

2.2 Preparing PCR

- Master mixes for different numbers of PCR's with a final reaction volume of 50 µl

1x	2x	3x	**Reagents**
35.5 µl	71 µl	106.5 µl	ddH_2O
2.5 µl	5 µl	7.5 µl	Sense primer (10 pmol/µl)*
2.5 µl	5 µl	7.5 µl	Antisense primer (10 pmol/µl)*
5 µl	10 µl	15 µl	10x buffer (Roche)
1 µl	2 µl	3 µl	dNTPs
0.5 µl	1 µl	1.5 µl	Roche (1U/µl)

4x	5x	6x	**Reagents**
142 µl	177.5 µl	213 µl	ddH_2O
10 µl	12.5 µl	15 µl	Sense primer

10 µl	12.5 µl	15 µl	Antisense primer
20 µl	25 µl	30 µl	10x buffer
4 µl	5 µl	6 µl	dNTPs
2 µl	2.5 µl	3 µl	Roche Taq (1U/µl)

- Aliquot 47 µl of master mix to each 0.5 ml thin-walled PCR tube and add two drops of mineral oil (approximately 50 µl).

- Add 3.5 cDNA template last in a separate lab. Label and centrifuge the tubes.

- Always include a negative control (ddH_20) and a positive control (a cDNA template which has amplified previously in PCRs performed under the same reaction conditions), when PCR is being used for diagnostic purposes.

8.4 Appendix B4

STANDARD OPERATING PROCEDURE FOR AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS IN THE EXOTIC DISEASES LABOROTORY

AGAROSE GEL ELECTROPHORESIS.

The working protocol describes the confirmation of the expected nucleic acid fragment using agar gel electrophoresis.

1. MATERIALS AND EQUIPMENT

1.1 Material

- Agarose (Roche)
- 1x TAE buffer (see 3 for preparation)
- 10 mg/ml Ethidium bromide (Promega)
- 100 bp DNA ladder marker (Promega)
- loading buffer
- Surgical gloves

1.2 Equipment

- Micro-pipette
- Micro-pipette tips

- Microwave oven
- Electrophoresis tank (Embi tec Cell)
- UV transilluminator

2. METHOD

- Make up a 1.5% agarose gel stock as follows:

  Weigh 1.5 g Multi purpose Agarose (Roche) in a 250 ml glass flask.

  Measure 100 ml of 1 x TAE and add 10 ml of ddH2O. Melt agarose completely by placing in the microwave on highest setting for 2 to 3 minutes. Gently swirl the mixture every minute. When the agarose is completely dissolved the mixture is clear. Adjust the volume back up to 100 ml by adding water, to compensate for any loss during the boiling process. Add 3 µl of ethidium bromide (EtBr)

  NB: Always wear gloves when working with EtBr as it is carcinogenic

- Measure 40 ml of the mixture and pour into the gel tray. Ensure that no air bubbles become trapped underneath the teeth of the comb. The gel is set when it becomes milky/opaque (30 min).

- Gently remove the combs. Place the tray in the electrophoresis tank and add 1xTAE until the gel and wells are completely submerged.

- Add 5 µl of 1x loading buffer to each 50 µl PCR sample and load 25 µl in the wells.

  The loading buffer contains bromophenol blue and xylene cyanol. The dye is used to track migration during electrophoresis.

- Load 5 µl of a 100 bp molecular weight maker

- Connect the cover and electrophoreses at 100V for 15 - 20 minutes

- Visualize on the UV transilluminator.

3. STOCK SOLUTIONS

1x TAE

4.84 g Tris base

1142 µl Acetic Acid

2000 µl 0.5M EDTA

Dissolve in ddH_2O and make up to 1000 ml

4. READING THE RESULTS

If FMDV is present in the specimen, an amplification of the expected fragment will be present. Correct product size estimation against a molecular weight marker will be confirmed.

The control samples:

The positive control must give an amplification of the correct size.

The negative control sample must be clear.

8.5 Appendix B5

STANDARD OPERATING PROCEDURE FOR PREPARATION OF PRIMARY PIG KIDNEY CELL CULTURES IN THE EXOTIC DISEASES LABOROTORY.

Primary porcine kidney (PK) cell cultures are used to isolate virus from field samples. A pig kidney is aseptically removed and placed in RPMI-1640 medium together with antibiotics, subsequently referred to as maintenance medium (MM) (78). The cortex is cut into small pieces (3 mm), transferred to a dimple flask and washed several times with 200 ml MM until the supernatant is clear. Pre-warmed 0.25 % trypsin (0.25 %; Merck) in PBS is added and the mixture stirred at 37 °C for 45 min. The pieces are allowed to settle, the supernatant collected and centrifuged at 1000 rpm for 5 min. The pellet is then resuspended in 10 ml MM with 2% foetal calf serum (FCS; Delta bioproducts) and stored on ice. Fresh Active Trypsin Versene is added to the pieces in the dimple flask and stirred continuously. It is incubated for a further 45 min. The abovementioned steps are repeated until no further desegregation is apparent. Active Trypsin Versene ATV) is made up as follows:

ACTIVE TRYPSIN VERSENE (ATV)

NaCl	160 g
KCl	8 g

$NaHCO_3$	11.6 g
D-Glucose	20 g
Trypsin (1:250)	10 g
EDTA	5 g
Phenol Red (0.5%)	8 ml
Add ddH_2O to a final volume of 1l	

The chilled cell suspensions are pooled, centrifuged once more and resuspended in 9 ml MM containing 10 % FCS [subsequently referred to as growth medium (GM)]. After performing a cell count, GM is added to obtain the correct concentration of cells and the cells are seeded onto 15 ml glass tubes. The tubes are incubated at 37°C for 48 hrs, rinsed with MM and 3 ml GM is added. When the cells are 90-100 % confluent, they are used for virus isolation.

8.6 Appendix B6

STANDARD OPERATING PROCEDURE FOR VIRUS ISOLATION USING PRIMARY PIG KIDNEY CELL CULTURES IN THE EXOTIC DISEASES LABOROTORY.

The working protocol describes the test to attempt to isolate virus from field or suspected Foot-and-mouth (FMD) outbreak samples from cloven hoof animals.

1. Definitions and abbreviations

CPE	cytopathic effect
ETOH	Ethanol (70%)
FCS	fetal calf serum
FMDV	Foot-and-mouth disease virus
l	liter
ml	millilitre
MM	maintenance medium
Monolayer	even layer of cells on surface of culture vessel
PBS	Phosphate buffer saline
rpm	revolutions per minute
TC	tissue culture
PKC	Pig kidney cell
u	units

2. Principle

Confluent monolayers of primary PKCC in tubes are used to isolate FMDV from field or suspected FMD outbreak samples received. The tissue samples are propagated onto PKCC's and observed under a converted microscope for the development of CPE for a period of 48 hours. If any virus is present, CPE will develop.

3. Materials and equipment

3.1 Apparatus

Balance (Satorius)

Roller-bottle apparatus (Leec)

- 86^{0}C freezer (Forma)

Walk-in + 37^{0}C incubator

1000 µl micro pipette (Gilson)

Inverted microscope (Olympus)

Laminar flow cabinet (Fibatron)

Roller-bottle baskets

Centrifuge (J-6B Beckman)

4^{0}C walk-in refrigerator

3.2 Consumables and equipment

10 ml test tubes

Blue + yellow pipette tips (Eppendorf-1000 µl + 100 µl)

5 ml glass syringe

10 ml glass syringe

4.5 ml Cryotubes (Nunc)

500 ml polyethylene beakers

Cotton wool to wipe bottles and surfaces with ETOH

20 ml glass bottles (Pyrex)

3.3 Reagents and cultures

i) Antibiotics Penicillin (1670 U/mg: 64g/litre) -Supplied by TC section

Neomycin (680µg/mg: 75g/litre) -Supplied by TC section

Streptomycin (787U/mg: 64g/litre -Supplied by TC section

Antibiotics are stored in -20^0C walk-in freezer

ii) Pig Kidney Cell Cultures

iii) 0.5% Citric acid (Merck) – in buckets in virus cubicles

Weigh out 0.5g Citric acid in a weighing boat

Dissolve in 20 ml distilled H_2O - add distilled H_2O to reach a final volume of 100 ml

iv) FCS (Delta Bioproducts)

v) Glyserol (Merck)

vi) MM - RPMI 1640 + 1 x antibiotics + 2% FCS

Add 2 ml FCS + 1 ml antibiotics to 1 l RPMI 1640 medium

vii) PBS: - Consist of 3 reagents:

i) PBS A

KCL	8 g			
$Na_2HPO_4.12\ H_2O$	115 g	or	Na_2HPO_4	45.43 g
NaCl	320 g			
$KH_2\ PO_4$	8 g			

Add distilled H_2O to a final volume of 2 l

ii) PBS B

$Ca\ Cl_2.6\ H_2O$	7.9 g

Add distilled H_2O to a final volume of 1 l

iii) PBS C

$Mg\ Cl_2.6\ H_2O$	4 g

Add distilled H_2O to a final volume of 1 l

Add 25 ml PBS b, 25 ml PBS c and 50 ml PBS a to distilled H_2O to reach a final volume of 1 l

vii) RPMI 1640 medium (Sigma:S6504))

ix) Sand (Merck)

4. Method

4.1 Safety and precaution

Sterile techniques must be observed and carried out throughout the duration of the assay. All the work must be carried out in a room destined for working with live FMDV. Addition of virus to the test tubes must be done in a clean, decontaminated laminar flow cabinet.

An interval of at least 20 minutes must be maintained between processing of different samples to prevent contamination.

The surface of the working area of the laminar cabinet must be decontaminated with acid in-between samples.

Materials that were in contact with live virus (e.g. pipette tips) must be deposited in a canister containing 0.5% Citric acid in H_2O.

4.2 Setting the roller- bottle apparatus

The apparatus is set at a speed of 1 revolution every 10 minutes

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4.3 Precision and sensitivity

4.3.1 Positive control

To ensure the susceptibility of the cells to virus, a known FMD virus laboratory vaccine strain SAR 9/81/1 (passage history BTY3BHK3B1BHK4B1BHK1RS3 – titre $\log_{10}$7.2) is used as positive control whenever the test is performed. This is done in a different laminar flow cabinet to prevent cross contamination with the sample to be tested.

4.3.2 Negative control

A negative cell control must always be included in the assay.

Follow the same procedure as for the positive control with the only difference that the cell cultures must be inoculated with medium only. This must be done in a separate laminar flow cabinet as the positive and the samples to be tested.

4.4 Propagation of the sample to cell cultures

- Confluent monolayer primary pig kidney cell cultures in 10 ml tubes are used.
- Remove the tissue sample out of the container it was sent in. Weigh off 1 g of the sample and add 5 ml PBS + 2 x antibiotics.
- Grind the sample with sand by means of a mortar and pestle and transfer to a 20 ml sterilized glass bottle.
- Centrifuge at 1000 rpm for 10 minutes and decant supernatant into a clean bottle. Keep on ice.
- Make a 1/10 dilution of each sample (4.5 ml RPMI- medium {2x antibiotics} + 0.5 ml sample; neat and 10^{-1}).
- Rinse tubes with cell cultures (10 per sample: 6 x neat and 4 x 10^{-1}) once with 1.5 ml serum-free RPMI-medium and add 0.2 ml of each dilution to the appropriate tubes, starting with the highest dilution.
- Roll the tubes for one hour at 37^0C.
- Decant the medium + virus and rinse the tubes twice with 1.5 ml medium; add 1.5 ml medium + 2% NBS and incubate (roll) for 24 hours at 37^0C.
- Inspect tubes microscopically for CPE, which, if positive, should show up by 24 hours. If no CPE is apparent after 48h, the cells must be passaged blind.
- Harvest the sample at 1000 rpm for 10 minutes to clarify. Decant the supernatant to a clean sterilised 10 ml bottle and 0.5 ml of the sample is given

out for typing and 0.2 ml for genetic characterisation. Add 2% sterilised Glyserol to the remainder of the sample and store in cryo-tubes at -70^0C until further use

- Results of all the tests as well as storage details is recorded and noted in the isolation log book

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