# VIROLOGICAL EXAMINATION OF UTERI FROM COWS WITH LOW FERTILITY USING EGG EMBRYO AND CELL CULTURE METHODS

by

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# INTRODUCTION

The problem of sterility is probably one of the largest and most perplexing conditions that confronts the dairy industry. Cows with impaired fertility are not efficient and therefore are not profitable for the dairy husbandman to retain in his herd. Sterility is second only to low milk production as a major reason for culling cows from the dairy herd (Asdell, 1951). The common infectious agents causing sterility such as Brucella abortus, Vibrio fetus and Trichomonas fetus, can be detected and controlled. It is the unknown causes of lowered fertility which are of great concern to the dairyman. Extensive research to determine the cause, or causes, of lowered breeding efficiency in dairy cattle is required if the problem is to be solved. Research programs have been organized to investigate the genetical, microbiological, physiological, and nutritional aspects of this problem (Pfau, 1957; Yates et al., 1957; Asdell and Mixner, 1957; and Bratton, 1957).

Casida (1953) defined a low fertility cow or a "repeat breeder" cow as a female that fails to conceive on the first service. He states that an average of 1.5 to 1.7 services for conception is satisfactory in dairy herds participating in artificial insemination programs in the United States. In contrast, he states that far too many conception rates average 2.0 to 3.0 services for conception which is not satisfactory.

Before the etiology of lowered fertility can be studied it must be established if this condition is primarily due to failure of fertilization of the ovum or due to a loss of the embryo. The rate of

fertilization of ova was considered to be high in dairy cattle in an investigation by Laing (1949). Kidder et al. (1952) estimated an overall fertilization rate of 86 per cent. He also estimated a fertilization rate of approximately 100 per cent if both the bull and cow were highly fertile. Assuming these fertilization rates to be accurate, reduced reproductive efficiency appears to be largely associated with failure of the fertilized ovum to continue to develop, i.e. embryonic death, and not with failure of fertilization of the ovum (Casida, 1953). Evidence to support the high incidence of embryonic death in dairy cattle and other animals will be given in the review of literature.

This investigation was conducted to attempt the isolation of a virus from dairy cows exhibiting a low fertility rate. Since the literature revealed conflicting and inconsistent results on bacteriological surveys it was felt that viruses could play an important part in lowered fertility. Little work has been done to determine if viruses are associated with infertility in cattle. Because of the effects of some viruses on the early developing embryo of other species (Gregg, 1941; Swan, 1944; Hamburger and Habel, 1947; Sautter, 1953; Shultz and DeLay, 1955) it was felt that they may be a contributing factor in the etiology of early embryonic deaths. Yates et al. (1958) stated:

> "Since sterility continues to be a problem in herds where all known infectious agents are controlled, it indicates that there are still unknown factors or agents that adversely effect conception."

They advised a continued search for bacteria, pleuropneumonia like organisms, and viruses using newer techniques.

# REVIEW OF LITERATURE

## Incidence of Embryonic Mortality

Hammond (1914) found that pregnant rabbits and pigs had shed many more ova than could be accounted for by the number of fetuses present in their uteri. He examined 38 rabbits, nine at nine days of pregnancy and the remainder at 23 and 25 days of pregnancy, and found 11 of them contained atrophic fetuses. In seven pregnant sows examined, four of them were found to have atrophic fetuses present. Later Hammond (1921) increased these data and on the basis of 22 pregnant sows found them to contain 67 per cent normal fetuses and 12 per cent atrophic fetuses with 20 per cent missing entirely. He also examined 80 pregnant ewes and found that only 87 per cent of the corpora lutea were represented by normal fetuses, and the remaining corpora lutea were represented by equal numbers of atrophic fetuses and missing fetuses.

Henning (1939) made a survey of pregnant ewes in the slaughterhouse. Using only the presence of dead fetuses or embryonic membranes as a criterion for his estimation, he found that 124 of 773 fetuses were dead. This was an embryonic death rate of 16.04 per cent. This estimate was considered to be low as it did not include possible earlier embryonic loss and resorption.

Brambell (1948) studying embryonic losses in the wild rabbit found that the loss between ovulation and parturition was no less than 43 per cent of ova ovulated. It was also determined that 10.2 per cent of these losses occurred before implantation and a great

majority of the remaining losses occurred before the middle of pregnancy.

A survey of pregnant sows in the packinghouse was made by Corner (1923), and he found an apparent embryo loss of 40 per cent in the first ten days of gestation. Sows examined in later stages of gestation showed only a 20 to 30 per cent embryo loss. He explained the discrepancy of per cent embryo losses by assuming that some sows were pregnant at an early stage, but at a later stage could not be recognized as having been pregnant because all of the embryos had died and complete resorption had occurred.

An embryonic death rate of 67.4 per cent in the first 25 days of gestation of "repeat breeder" sows was reported by Warnick et al. (1949). "Repeat breeder" gilts had an embryonic death rate of 23.9 per cent. These workers concluded that the major cause of sterility in gilts was attributed to fertilization failure and in sows it was due to embryonic death.

Laing (1949) reported that embryonic death was an important factor in lowered fertility in cattle. Over a two year period he conducted a study on low fertility heifers. The first year 24 heifers were inseminated. Six of them were killed 40.00 to 66.75 hours after insemination and all six had normal fertilized ova. Twelve of the remaining 18 heifers had not returned to heat by the 24th day post-insemination and were slaughtered at this time. Nine had normal embryos and three had no embryos. Two of the three with no embryos present had fetal membranes present in their uteri. The six heifers that had returned to heat were inseminated a second time and killed 43.75 to 71.00 hours later. All six had normal fertilized ova present.

The second years study was on 23 low fertility heifers. Seventeen became pregnant on the first service, three returned on or before the normal estral cycle, two returned after long estral cycles, and one failed to conceive after many estral cycles.

Tanabe and Casida (1949) made observations on 104 cows that had been bred four to thirteen times successively without conceiving. The cows in this study were all under 10 years of age, had had at least one calf, and genital abnormalities could not be detected by rectal palpation. Three days post-insemination 59 cows were slaughtered and 66.1 per cent of them had normal fertilized ova present. At 35 days post-insemination the remainder of the cows were slaughtered and only 23.1 per cent had normal embryos. The estimated embryonic death rate was 65.1 per cent. Six per cent of the cows had definite genital abnormalities that would prevent fertilization. A similar study on low fertility nulliparous heifers disclosed an estimated 54.1 per cent embryonic death loss by 30 days of pregnancy (Tanabe and Almquist, 1953).

The highest incidence of embryonic death seems to occur between the 16th and 34th day of pregnancy (Hawk et al., 1955). Fifty "repeat breeder" cows were slaughtered 16 days post-insemination, 29 of them had normal embryos. Fifty similar "repeat breeder" cows were slaughtered 34 days post-insemination and only 14 had normal embryos. The estimate of embryonic losses from 16 to 34 days of pregnancy was 51.7 per cent. An investigation by Fosgate and Smith (1954) revealed a fetal loss of 6.38 per cent between pregnancy diagnosis at 34 to 50 days and parturition.

Stewart (1952) estimated embryonic mortality to fall in a range from 15 to 44 per cent, based on records of return breeding in a number of dairy herds in Great Britain.

The role of the bull as the cause of embryonic death has been investigated. There was no evidence that more embryonic deaths occurred from use of low fertility bulls than from use of high fertility bulls (Kidder et al., 1954). However, it was noted in another study that certain low fertility bulls used on first service decreased the conception rate of cows when subsequently bred by bulls of high fertility (Christian and Casida, 1951). It was postulated that the low fertility bulls transmitted some type of infection to the cows when mated.

Studies of the effect of mating systems on embryonic mortality have been made. Christian et al. (1951) found no beneficial increase of conception rate in "repeat breeder" cows when inseminated with semen from bulls of other breeds. Hawk et al. (1955) analyzed 837 matings and reported that inbreeding tends to increase embryonic mortality but not to a significant degree.

## Bacteriology

The possible implication of uterine infections as a cause for lowered fertility has been studied with variable and inconsistent results. Hatch et al. (1949) incriminated bacterial infections as a cause of low fertility in cattle. A large percentage of cervical and vaginal samples taken from "repeat breeder" cows revealed infections with gram positive rods, similar to Corynebacterium bovis and Coryne-

bacterium renale, and gram positive cocci.

According to Easley et al. (1951) the incidence of uterine infections in "repeat breeder" cows and apparently normal cows was 80 per cent and 60 per cent respectively. Micrococcus pyogenes was found in 45 per cent of "repeat breeder" cows but only in 26 per cent of normal cows.

Watts (1952) stated that infections with Corynebacterium and Streptococcus were an important cause of lowered fertility in cattle. He remarked, however, that the role of these organisms in infertility was not clear.

Lindley and Hatfield (1952) isolated organisms of the genera Micrococcus, Neisseria, Corynebacterium, Achromobacter, Gaffkya, and Streptococcus from "repeat breeder" cows. They noted that the genus Neisseria was markedly evident in this survey.

A bacteriological study by Gunter et al. (1955) explored the role of infection as a cause of lowered fertility. Two-hundred sixty uterine swab samples from 106 dairy animals were taken. Samples from normal breeding cows showed 33 per cent were not infected whereas only five per cent of samples from repeat breeders were not infected. The predominate organisms isolated were Streptococcus, strains of Micrococcus pyogenes and Corynebacterium. Saprophytic strains of these organisms were dominate in normal cows while pathogenic strains were dominate in difficult breeders.

Gibbons (1957) indicated that Streptococcus and Staphylococcus organisms were associated with varying degrees of endometritis in cattle. He infers that these organisms could be a cause of lowered fertility.

Kiesel and Dacres (1959) cultured the external ora of cervices of cattle. Only 383 culture of 1,359 cultures taken showed the presence of bacteria. It was felt by these investigators that most of the bacteria isolated were transient opportunists. They also infused suspensions of pathogenic bacteria into the genital tracts of clean heifers and these apparently had no effect on their breeding efficiency. Routine intrauterine infusions of antibiotics did not affect breeding efficiency of the cattle. They concluded that non-specific bacterial infections were not a major cause of infertility.

Bacteriological studies of 157 cows by Martinez (1955) revealed a high incidence of infection in low fertility cows. One-hundred of the cows conceived on the first insemination and 83 of these were infected with bacteria. All 57 cows that did not conceive on the first service were infected. Micrococcus pyogenes was the dominant organism cultured from all cows.

## Virology

Viruses and viral infections have been shown to affect the early development of embryos by causing developmental abnormalities and death of the embryo. The fact that mothers having rubella in the early stages of pregnancy may give birth to infants with congenital cataracts and congenital heart disease was first reported by Gregg (1941). Swan (1944) reported on three cases in which women had contacted rubella in the second month of pregnancy. All three babies were born with congenital heart defects and two of the three babies were born three weeks premature and had congenital cataracts. All

three babies died before 10 weeks of age. Erickson (1944) reported on 11 cases of mothers who had rubella from two weeks to two and one-half months of pregnancy. All of the babies born were small, difficult to feed and had congenital cataracts. Two of the babies had congenital heart defects. He concluded that 100 per cent of babies will have abnormalities if the mother has rubella in the first two months of pregnancy.

Teratogenic and lethal effects of influenza-A and mumps viruses inoculated into 48 hour chick embryos were reported by Hamburger and Habel (1947). Influenza-A virus caused microcephaly, micrencephaly, axial flexion and impaired growth of the chick embryo. Embryos seemed to be much more susceptible to infection with influenza-A and mumps virus at this early age than they were when infected at later stages of development. Using neutralization tests Shear et al. (1955) confirmed that these teratogenic and lethal effects were actually produced by infection with influenza-A virus. Heath et al. (1956) demonstrated teratogenic and lethal effects in the two day old chick embryo with infection by Herpes simplex, vaccinia, and influenza-A (strain NWS) viruses. They also showed a lethal effect of distemper virus in early embryos, whereas death does not occur when older embryos are infected with this virus.

Developmental abnormalities, followed by death of the embryo, were produced in early chick embryos with Newcastle disease virus infections (Blattner and Williamson, 1951; Williamson et al., 1953; and Robertson et al., 1955). These workers showed that developmental abnormalities occurred most frequently in chick embryos inoculated

from 48 to 60 hours of age. Seventy-two hour embryos had only a few defects and 82 hour embryos had no defects. It was also noted that the youngest embryos experienced the highest rate of mortality from the Newcastle disease virus infection.

When sows are vaccinated with modified live hog cholera virus vaccine between the 10th and 16th day of pregnancy there is a high rate of fetal abnormalities produced (Sautter et al., 1953 and Young et al., 1955). Sows injected with modified live hog cholera virus at 10 to 16 days of gestation had a 38 per cent fetal abnormality rate when slaughtered at 70 to 105 days of pregnancy. Uninoculated controls showed a fetal abnormality rate of three per cent (Young et al., 1955).

Shultz and DeLay (1955) reported a high incidence of losses in newborn lambs when ewes were vaccinated with modified live bluetongue virus between the fourth and eighth week of pregnancy. They were associated cerebellar and cerebral abnormalities in this report of lamb losses.

Only one report of a virus being incriminated in low fertility and embryonic mortality was found in the literature. Millar (1955), while studying herd infertility problems, noted apparent infective forms of infertility characterized by two types of syndromes. The first type was herd infertility with low conception rates, post-coital genital discharges, irregular estrous cycles, and evidence of early fetal deaths and abortions. The second type of herd infertility was associated with rapid widespread vaginitis and genital discharge. The onset was not related to coitus and maiden heifers showed signs a few days after appearance of the syndrome in adult females. Millar

found that upon inoculation of bacteria free genital mucus onto the chorioallantoic membrane and into the yolk sac of chick embryos, a thickening of the chorioallantoic membrane was noted and yolk sacs were coagulated. Serial passages in the yolk sac and on the chorioallantoic membrane did not produce death of the chick embryo. Third passage chorioallantoic and third passage yolk sac material was inoculated intravaginally into two heifers with a resultant edematous endometritis and genital discharge. The chorioallantoic material seemed to be more potent in inducing vetibular vaginitis. Millar felt that the vaginitis was a frequent, but not a signficiant, factor in the lowered fertility of cows. He postulated that he was working with two infectious agents, one causing vaginitis and one causing an endometritis.

## MATERIALS AND METHODS

### Chick Embryo Inoculation

The advantages of using the chick embryos for the cultivation of viruses are as follows: (1) They lack latent viral infections. (2) They are incapable of producing antibodies. (3) They are convenient and easy to inoculate. (4) They show unequivocal changes or symptoms which make viral infection easily recognized. (5) They have proven to support the growth and multiplication of a wide range of animal viruses. The disadvantage of using the chick embryo for viral cultivation becomes more evident in modern virology. Even though many viruses multiply in the chick embryo, a large number will not. For this reason, it would not be adequate to use the chick embryo alone

in a survey attempting the isolation of a viral agent (Beveridge and Burnet, 1946).

Three routes of chick embryo inoculation were used: (1) the yolk sac, (YS), (2) the allantoic cavity, and (3) the chorioallantoic membrane (CAM).

Yolk Sac Inoculation. Five or six day old embryonated eggs were used for yolk sac inoculation. The blunt end of embryonated eggs were swabbed with seven per cent tincture of iodine. After swabbing, a hole was punched in the shell in the center of the blunt end of the eggs. A 1 ml tuberculin syringe with a 1½ inch 20 gauge needle was used for inoculation. The needle was directed 1 to 1¼ inches down the long axis to the center of the eggs and .25 ml of the sample was inoculated. The eggs were candled daily for deaths. Eggs that died up to the second day after inoculation were considered to be traumatic deaths.

Deaths occurring after this time were checked for bacterial contamination and the yolk sacs saved. If either a stained smear of the yolk material or a nutrient broth culture indicated bacterial contamination, the yolk sacs were discarded. The yolk sacs were harvested 7 to 9 days after inoculation. This was done by swabbing the blunt end of the eggs with seven per cent tincture of iodine and removing the shell above the air sac with curved dissecting scissors. The air sac membranes and chorioallantoic membranes were removed with thumb forceps. The yolk sacs were grasped by the yolk stalk with thumb forceps and a portion of each put into a sterile 100 ml prescription bottle which contained 10 ml of sterile phosphate buffer solution

and ground glass. A small section of the yolk sacs were smeared on a slide to be stained later with Giemsa's stain. The harvested yolk sacs were ground by shaking them in the prescription bottle. The ground yolk sac material was decanted into sterile screw cap vials and stored at -20° C until the material was used for another passage.

A 1:10 dilution of the harvested yolk sac material was used as inoculum to make the next serial passage in yolk sacs.

Allantoic Cavity Inoculation. Ten or eleven day old embryonated eggs were used for allantoic cavity inoculation. The eggs were candled and the edge of the air sacs were marked in an area which was free of blood vessels and would allow entrance of the needle into the allantoic cavity with a minimum amount of trauma. The position of the embryos was also marked. The shells were swabbed with seven per cent tincture of iodine. Using a carborundum disc, holes were drilled in the shells where they had been previously marked. A 1 ml tuberculin syringe with a ½ inch 26 or 27 gauge needle was used to make the inoculations. The needle was directed into the allantoic cavity away from the embryo, and .25 ml of inoculum was deposited in the allantoic cavity of each egg. Eggs were candled daily for deaths. Deaths occurring up to 24 hours were considered traumatic. Deaths occurring after this time were checked for bacterial contamination and the fluid saved. If a stained smear or nutrient broth culture showed that death was due to bacterial contamination, the fluid was discarded.

Harvesting of the fluid was accomplished seven to nine days after inoculation. The blunt end of the eggs were swabbed with seven per cent tincture of iodine and the shells above the air sac removed by

cutting with sterile curved dissecting scissors. The air sac membranes and chorioallantoic membranes were lifted away with sterile thumb forceps. Sterile 1 ml pipettes were used to remove the fluid. The fluids were transferred into sterile screw cap vials and stored at -20° C until they were used as inoculum for another passage. Inoculum for a subsequent passage was prepared by diluting the allantoic fluid 1:10 in phosphate buffer solution.

Chorioallantoic Membrane Inoculation. Ten or eleven day old embryonated eggs were used for chorioallantoic membrane inoculation. The eggs were candled and a point was marked on the side of the eggs where the chorioallantoic was best developed and in an area that was free of blood vessels. The blunt end of the eggs and the area marked upon candling were swabbed with seven per cent tincture of iodine. Holes were punched in the shells above the air sac as previously described. A carborundum disc was used to drill a hole in the shells at the point where they had been marked on the side, being careful not to break the shell membranes or injure the chorioallantoic membranes. A drop of sterile saline was put on the shell membranes exposed by the drilled hole. A needle point was used to slit the shell membranes, being careful not to penetrate the chorioallantoic membrane, which lies immediately beneath the shell membrane. The slit in the shell membranes allowed the drop of saline to run between the shell membranes and the chorioallantoic membranes and act as a fluid wedge for the separation of the chorioallantoic membranes from the shell membranes. A rubber bulb was applied to the hole over the air space. Gentle suction to produce a negative pressure in the air

space caused the chorioallantoic membrane to separate and pull away from the shell membrane.

With a 1 ml tuberculin syringe and a ½ inch 26 or 27 gauge needle, the inoculations were performed by introducing the needle through the holes over the dropped chorioallantoic membranes and depositing .25 ml of inoculum on the surface of the membranes. Scotch tape was used to cover the holes in the shells and the eggs incubated with the blunt end up.

Deaths occurring in 24 hours were considered traumatic. Deaths after this time were checked for bacterial contamination by a stained smear or nutrient broth culture and the chorioallantoic membrane checked for lesions of viral infection. After 7 to 9 days of incubation the membranes were harvested. The pointed end of the eggs were removed by cutting with sterile curved dissecting scissors and the embryo, yolk sac, and fluids allowed to fall from the eggs. This left the chorioallantoic membranes adherred to the shells. The membranes were peeled from the shells with sterile thumb forceps, dropped into a petri dish containing sterile saline and washed to remove blood and egg albumin. The membranes were then transferred to a sterile dry petri dish and spread with sterile thumb forceps. They were observed against a black background for focal lesions on the membranes which might indicate viral infection. The membranes were also checked for lesions under a dissecting microscope at a magnification of 60X. After examination the membranes were transferred into sterile screw cap vials and stored at -20° C.

Membranes were prepared for a serial blind passage by freezing and thawing rapidly three times in a mixture of dry ice and ethyl alcohol. The supernate was diluted 1:10 in phosphate buffer solution and used as inoculum.

Staining of Yolk Sac Smears. Stock Giemsa's staining solution was prepared by dissolving 1 gm of Giemsa dry powder to 66 ml of glycerol at 55° to 60° C for 1.5 to 2 hours. To this 66 ml of acetone free absolute methyl alcohol was added. This solution was dispensed into small dropper bottles and sealed tightly. The staining solution consisted of: 10 ml methyl alcohol (acetone free), 1½ ml stock Giemsa's staining solution, 5 drops 0.6 per cent $NaHCO_3$, and 100 ml neutral distilled $H_2O$. Yolk sac smears fixed with methyl alcohol were allowed to remain immersed in the staining solution overnight. The slides were cleared of excess stain by allowing them to stand in acetone for 3 minutes, then in acetone (60 parts) and xylol (40 parts) for 3 minutes, and a final clearing in xylol for 3 minutes. The slides were allowed to air dry and were examined under oil at a magnification of 970X.

## Cell Cultures

The advantages of using cell cultures for isolation of viral agents in conjunction with this survey were as follows: (1) They provide homologous as well as heterologous cells. (2) Cell cultures are relatively free of antibodies, assuming that the serum used is of another species and either low concentration or no serum is used. (3) Cytopathogenic changes, often reflecting the presence of virus,

eliminates tedious, costly, and often discouraging blind passages in embryonated egg and intact animals (Madin, 1959).

Preparation of Glassware. For optimum growth of cell cultures in contact with glass culture vessels required that the surface be clean and free of toxic material. Glassware for cell cultures was cleansed separately from other routine washing of laboratory glassware to meet this requirement. To accomplish thorough cleansing of the glassware the following protocol was rigidly followed and routinely yielded suitably cleansed glassware.

All dirty glassware was rinsed and immersed in a solution of one per cent sodium lauryl sulfate until it was washed. Glassware was washed to remove all cells and medium from the surface. The glassware was then completely immersed in a one per cent sodium lauryl sulfate solution and autoclaved for 30 minutes. Following autoclaving the glassware was rinsed thoroughly with tap water, followed by three rinses in distilled water and one rinse in double distilled water. The glassware was air dried before sterilization in either a hot air oven or an autoclave.

Phosphate Buffer Solution (PBS). Water used in the preparation of this and other types of media was double distilled. The second distillation was carried out in an all glass still.

Solution A. 16 g NaCl, .4 g KCL, 2.3 g $Na_2HPO_4$, .4 g $KH_2PO_4$ were dissolved in 1500 ml $H_2O$.

Solution B. .2 g $MgCl \cdot 6H_2O$ was dissolved in 200 ml $H_2O$.

Solution C. .2 g $CaCl_2$ was dissolved in 200 ml $H_2O$.

Solutions A, B, C, and a flask containing 200 ml of $H_2O$ were autoclaved for 20 minutes. The solutions were then set in the refrigerator until cooled. When the solutions were cold they were mixed by adding solutions B and C to solution A. The sterile water was used to bring the volume up to 2000 ml. Five hundred thousand units of crystalline penicillin (Pfizer) and 200,000 $\mu$g of streptomycin sulfate (Pfizer) were added to the mixture. The mixture was distributed, aseptically, into sterile 160 ml prescription bottles, appropriately marked and stored at 4° C for use.

PBS Without Calcium and Magnesium (PD). PD was solution A of the PBS, alone, made up to a volume of 2000 ml. It was autoclaved 20 minutes, cooled in the refrigerator and 500,000 units of penicillin and 200,000 g of streptomycin added. This was distributed in sterile 160 ml prescription bottles, appropriately marked, and stored at 4° C for use.

Eagle's Minimum Essential Medium (MEM). This medium is conveniently made into a series of concentrates which can be stored at -20° C. Double distilled water was used in all preparations. Phenol red was used as a pH indicator and was added in stock solution 1.

Stock Solution 1 (Salts plus glucose, 10 fold concentrate): 68.0 g NaCl, 4.0 g KCl, 2.0 g $CaCl_2$, 2.0 g $MgCl_2 \cdot 6H_2O$, 1.1 g $NaH_2PO_4 \cdot 1H_2O$, 10.0 g glucose, and 0.2 g Phenol red dissolved in 1000 ml $H_2O$ and distributed in 100 ml aliquots for storage at -20° C.

Stock Solution 2 (Amino Acids, 100 fold concentrate): In preparation of amino acid solutions only l-amino acids were used; if only dl-amino acids were available, twice the given concentration was

used. 10.5 g arginine, 3.1 g histidine, 5.2 g isoleucine, 5.8 g lysine, 1.5 g methionine, 3.2 g phenylalinine, 4.8 g threonine, 1.0 g tryptophane, and 4.6 g valine dissolved in 1000 ml $H_2O$. These amino acids had to be dissolved by heating to 80° C. They were distributed in 50 ml aliquots and stored at -20° C.

Stock Solution 3 (Amino acid, 100 fold concentrate): 2.4 g cystine and 3.6 g tyrosine were dissolved in 100 ml double distilled $H_2O$ by adding 9 ml concentrated HCl and heating to 60-80° C. When cooled the solution was distributed into 50 ml aliquots and stored at -20° C.

Stock Solution 4 (Amino acids, 100 fold concentrate): 29.2 g glutamine dissolved in 1000 ml $H_2O$, distributed in 50 ml aliquots, and stored at -20° C.

Stock Solution 5 (Vitamins, 1000 fold concentrate): 1.0 g choline, 1.0 g pryridoxal, 0.1 g riboflavin, 1.0 g thiamine, 2.0 g inositol, 1.0 g nicotinamide, and 1.0 g sodium pantothenate dissolved in 1000 ml $H_2O$, distributed in 50 ml aliquots, and stored at -20° C.

Stock Solution 6 (Vitamins, 100 fold concentrate): 0.1 g folic acid dissolved in 1000 ml $H_2O$ by adding a few drops of 0.5 N NaOH. It was distributed in 50 ml aliquots and stored at -20° C.

Stock Solution 7 ($NaHCO_3$, 20 fold concentrate): 40.0 g $NaHCO_3$ dissolved in 100 ml $H_2O$ and sterilized by filtration through a 0.45 $\mu$ pore size Millipore filter assembly. This solution was then distributed in 100 ml aliquots and stored at 4° C.

The Complete Medium: The complete medium was prepared in the following manner. Stock solutions were thawed and the following volumes mixed in a 2 liter flask: 100 ml Stock Solution 1, 300 ml double

distilled $H_2O$, 10 ml Stock Solution 2, 10 ml Stock Solution 3, 10 ml Stock Solution 4, 1 ml Stock Solution 5, 10 ml Stock Solution 6, 200 ml double distilled $H_2O$ and 50 ml Stock Solution 7. Five hundred thousand units penicillin and 200,000 mcg streptomycin, were added and the volume made up to 1000 ml with double distilled $H_2O$. This preparation was filtered through a Millipore filter assembly using a 0.45$\mu$ pore filter pad. After filtration, 10,000 units of Mycostatin sterile powder (E. R. Squibb & Sons) was added. This preparation was distributed in 100 ml and 200 ml volumes and stored at -20° C.

Serum. Two types of serum were used for growth and maintenance of cell cultures. Calf serum was used for the growth of cells until confluent monolayers of cells were obtained. Horse serum was used in the maintenance medium for inoculated cultures and stock cultures.

Calf Serum: Blood was collected aseptically from health calves in sterile 1000 ml glass cylinders and allowed to clot and set approximately 48 hours. The serum was pipetted off aseptically and distributed in 200 ml volumes. Each 200 ml volume was checked for contamination by inoculating thioglycollate broth medium and incubating for 7 days at 37° C. The serum was stored at 4° C until used.

Horse Serum: Horse serum was obtained from the Colorado Serum Company. It had been tested for sterility, toxicity, and suitability for use in cell culture medium.

Growth Medium: Growth medium consisted of 85 per cent Eagle's MEM and 15 per cent calf serum. This medium was used for growth of the cells until a confluent monolayer of cells was obtained.

Maintenance Medium. Maintenance medium contained 99 per cent Eagle's MEM and one per cent horse serum. This medium was used to maintain cells in a monolayer after inoculation with virus or unknown uterine material and for maintaining stock cultures of cells. Horse serum was used to lessen the chance of the serum containing neutralizing antibodies against any virus that might be present in the inoculum.

Trypsin Solution. 2.5 g trypsin (1-300, Nutritional Biochemicals Corporation) was added to 1000 ml sterile PD solution. Five hundred thousand units of penicillin and 200,000 mcg streptomycin were added. The trypsin was allowed to dissolve overnight at 4° C. The trypsin solution was sterilized by filtration with a Millipore filter assembly using a 0.45 $\mu$ pore size filter pad. This solution was distributed in 50 ml and 100 ml volumes and stored at -20° C.

Preparation of Bovine Kidney Cell Cultures. Methods for preparing bovine kidney cell cultures were modifications of those described by Dulbecco and Vogt (1954) and those described by Madin et al. (1957). Kidneys from three week old to three month old healthy calves were used. The kidney from a euthanized calf was removed aseptically in the following manner. (1) The hair was removed from the paralumbar fossa on the right side by shaving with a straight razor. (2) The area was scrubbed with soap and water to remove dirt and bacteria from the area. (3) A six inch incision was made through the skin, fascia, abdominal muscles, and peritoneum with a sterile scalpel. (4) The incision was held open with sterile Allis tissue forceps. (5) The capsule of the right kidney was grasped with sterile forceps and the bessels and connective tissue attachments cut with a sterile scalpel.

(6) The kidney, enclosed in its capsule, was removed and placed in a sterile beaker containing PBS.

The kidney was transferred to an isolation booth and the preparation of the cells for culture was conducted in the following sequence. (1) The kidney was transferred from the beaker to a sterile 100 mm petri dish containing PBS. (2) The capsule was removed with the aid of sterile thumb forceps and scissors. (3) Portions of the renal cortex were excised with sterile curved dissecting scissors and placed in another sterile 100 mm Petri dish containing cold PD. About 35 grams of the cortical tissue was removed. (4) The cortical tissue was transferred to a sterile dry 100 mm Petri dish, and minced with two sterile scalpels until the pieces of tissue were three to five mm in size. (5) The minced tissue was spooned into a sterile 300 ml Bellco trypsinizing flask designed for use with magnetic stirrers. The flask had four flutes, a dam in the neck to prevent tissue from being poured off with the cell suspension, and a side arm pour-ff spout. (6) Fifty ml of cold PD was added to the tissues and the kidney-PD mixture agitated for one minute. The supernate was decanted from the minced tissue. (7) Fifty ml of warm PD was added and the mixture agitated another minute. The purpose of the two washings with PD was to remove the red blood cells from the tissues. (8) After decanting the warm PD from the second washing, 50 ml of warm 0.25 per cent trypsin was added and the kidney-trypsin mixture agitated for 15 minutes. The supernate was discarded. This first trypsinization was for further removal of red blood cells and damaged cells. (9) Another 50 ml of warm 0.25 per cent trypsin was added and the kidney-trypsin

mixture agitated 15 to 20 minutes. At the end of this time the suspension of cells was decanted into a sterile graduated 50 ml conical centrifuge tube that contained 5 ml sterile calf serum. The serum was present to stop the action of the trypsin. (10) A second 15 minute trypsinization was done as in step 9. As a rule two trypsinization processes yielded all of the cells required. (11) The cell suspensions were centrifuged for two minutes at 600 rpm and the trypsin poured from the packed cells. (12) The packed cells were resuspended in 10 ml of growth medium and pipetted gently to disperse the clumps of cells. (13) The suspended cells were diluted 1:200 by volume in growth medium and were dispensed into the proper glass vessels for growth. Fifteen ml of the cell suspension was used for seeding milk dilution bottles, 2 ml for 16 x 150 mm tubes, and 5 ml for 60 mm Petri dishes.

The cells were incubated at 37° C in an atmosphere of flowing air and five to seven per cent $CO_2$. Cells in test tubes were incubated in racks that were tilted at a five degree angle. Cells were left undisturbed for 48 hours to allow them to attach to the glass surface. The growth medium was changed at 48 or 72 hours. It took five to seven days for a confluent monolayer of cells to grow. At this time the cells were ready for inoculation, transfer, or maintenance as stock cultures. Regardless of their use, the growth medium was changed to a maintenance medium.

Secondary Cultures. Secondary cultures were obtained from the primary cultures. These cells seemed to adhere to glass for a longer period of time and contained less debris than did the primary cultures. For this reason secondary cultures were used for experiments in which

the cells were held for two to three week periods. The cell morphology of secondary cultures were indistinguishable from that of primary cultures. Secondary cultures up to four passages were used before new primary cultures were obtained.

The procedure for transferring cells was as follows: (1) Medium was decanted from stock culture bottles. (2) The cell layers were washed two times with 10 ml of warm PD. (3) The cell layers were rinsed with 5 ml of warm 0.25 per cent trypsin. (4) Five ml of warm 0.25 per cent trypsin was added to the cell layer and allowed to incubate at 37° C until the cells became completely detached from the glass surface. The time required for detachment of the cells varied from 10 minutes to 30 minutes. (5) The cell-trypsin suspension was gently pipetted to break up the clumped cells and the suspension of cells transferred to tubes for centrifugation. (6) The cells were centrifuged for two minutes at 600 rpm, the trypsin supernate decanted off, and the cells resuspended in growth medium. (7) The packed cell volume was diluted 1:200 in growth medium and dispensed into tubes and stock culture bottles. Incubation of these cultures was the same as previously described for primary cultures.

Fixation and Staining of Cell Cultures. Cell layers were stained to observe possible nuclear degeneration or vacoulization of the cytoplasm. Cells were fixed with 10 per cent neutral formaldehyde and stained with Giemsa's stain.

Ten per cent Neutral Formaldehyde: To neutralize the formaldehyde, 37 per cent formaldhyde solution was saturated with magnesium carbonate. Excess $MgCO_3$ was filtered off to give a stock solution

37 per cent neutral formaldehyde solution. Ten per cent neutral formaldehyde was made by diluting 250 ml of 37 per cent neutral formaldehyde with 750 ml PBS.

Giemsa's Stain: One gram Giemsa's stain, dry powder, was mixed with 66.0 ml glycerine and maintained at 55 to 60° C for 1½ to 2 hours. Sixty-six ml methyl alcohol was added to make the Giemsa stain concentrate. For use this concentrate was diluted 1:4 in distilled water.

The procedure for fixing and staining cells was as follows. (1) The growth medium was poured off and the cells rinsed once with PBS. (2) Cells were fixed for one minute with 10 per cent neutral formaldehyde. (3) The fixing solution was discarded and the cells rinsed once with tap water. (4) Giemsa's staining solution was added to cover the cells and 15 minutes allowed for staining. (5) The stain was discarded and the cells rinsed with tap water. (6) The cells were allowed to air dry. Upon drying the cells were ready for examination. Cells so fixed and stained will remain on the glass indefinitely without deterioration. These cells may be kept as a permanent record.

## Collection of Samples

Samples for virological cultures were obtained from test cows at slaughter. When the carcass was eviscerated the uterus was excised. One of the horns was ligated, if the animal was pregnant the non-pregnant horn was the one ligated. The ligated horn was removed and an area on the greater curvature of the horn was swabbed with seven per cent tincture of iodine. A sterile 10 ml syringe and 20 gauge

1½ inch needle was used to inject 10 ml of sterile peptone buffered water into the lumen of the uterus. The horn was massaged to agitate the peptone buffered water and it was withdrawn into the syringe. This material was placed in a sterile test tube for transfer to the laboratory. The peptone buffered water sample and uterine horn were placed in an ice chest for transfer to the laboratory at 0° C. Upon arrival at the laboratory the uterus was opened aseptically by first searing the surface with a heated spatula and making a four inch incision with a sterile razor blade. Uterine mucosa was cut from the uterine lumen with a sterile curved biopsy scissors. The mucosal tissue was transferred to a sterile mortar and pestal and sterile sand added. The mucosal tissue was ground into a fine paste and peptone water added to suspend the ground tissue. The tissue homogenate was transferred to a sterile test tube. The uterine washing taken at the packinghouse and the mucosal tissue homogenate were pooled to be used as inoculum for chick embryos and bovine kidney cell cultures. Samples were stored at -20° C until used.

## EXPERIMENTAL PROCEDURES AND RESULTS

Over a nine month period uterine samples from 47 dairy cows were cultured for presence of viruses. Thirty-five of these cows were from the Kansas State University dairy herd, eight were slaughterhouse cows from the Armour Packing Company in Kansas City, and four were from the Garden City Experiment Station dairy herd. Complete breeding histories were available on the 35 cows from the Kansas State University dairy herd. Limited or no breeding histories were available for the remaining cows in the study.

Fifteen uterine samples were collected from "repeat breeder" cows that had been bred two or more times without conceiving and were non-pregnant at time of slaughter (Table 1). Three uterine samples were

Table 1. Samples from "repeat breeder" cows not pregnant at slaughter.

| Sample No. | No. of previous calves | No. of heat cycles since last parturition | No. of services since last parturition | No. of days since last service |
|---|---|---|---|---|
| 405 | 2 | 3 | 3 | 2 |
| 407 | 2 | 6 | 4 | 42 |
| 411 | 0 | 2 | 2 | 6 |
| 412 | 5 | 7 | 7 | 32 |
| 424 | 2 | 1 | 1 | 120 |
| 445 | 6 | 5 | 4 | 12 |
| 456 | 0 | 16 | 14 | 136 |
| 459 | 1 | 7 | 6 | 55 |
| 460 | 1 | 10 | 8 | 29 |
| 462 | 3 | 7 | 6 | 37 |
| 463 | 5 | 5 | 4 | 22 |
| 467 | 1 | 3 | 2 | 6 |
| 468 | 1 | 4 | 3 | 39 |
| 469 | 0 | 12 | 11 | 10 |
| 482 | 2 | 7 | 6 | 87 |

collected from cows with repeat breeding histories and were pregnant less than 35 days at time of slaughter (Table 2). The embryos from

Table 2. Samples from cows with repeat breeding histories but pregnant less than 35 days at slaughter.

| Sample No. | No. of previous calves | No. of heat cycles since last parturition | No. of services since last parturition | No. of days pregnant |
|---|---|---|---|---|
| 401 | 1 | 4 | 3 | 25 |
| 461 | 0 | 11 | 10 | 19 |
| 471 | 1 | 5 | 4 | 35 |

these cows were still in the stage of development in which the highest incidence of embryonic mortality occurs. Fourteen uterine samples were taken from cows with histories of two or more services before conception and they were pregnant more than 35 days at time of slaughter (Table 3). Three uterine samples were obtained from cows with apparently

Table 3. Samples from cows with repeat breeding histories but pregnant more than 35 days at slaughter.

| Sample No. | No. of previous calves | No. of heat cycles since last parturition | No. of services since last parturition | No. of days pregnant |
|---|---|---|---|---|
| 402 | 1 | 4 | 4 | 150 |
| 404 | 2 | 7 | 4 | 256 |
| 443 | 5 | 17 | 10 | 51 |
| 444 | 2 | 2 | 2 | 68 |
| 446 | 2 | 4 | 4 | 117 |
| 448 | 2 | 5 | 5 | 56 |
| 465 | 3 | 4 | 3 | 111 |
| 481 | 0 | 7 | 7 | 64 |
| 483 | 1 | 3 | 2 | 151 |
| 484 | 1 | 8 | 6 | 177 |
| 485 | 1 | 6 | 4 | 168 |
| 491 | 1 | 3 | 3 | 60 |
| 492 | 1 | 3 | 3 | 48 |
| 493 | 2 | 3 | 3 | 189 |

normal breeding histories that conceived on the first service and were maintaining pregnancy at the time of slaughter (Table 4). Sample

Table 4. Samples from cows conceiving on first service.

| Sample No. | No. of previous calves | No. of heat cycles since last parturition | No. of services since last parturition | No. of days pregnant |
|---|---|---|---|---|
| 413 | 1 | 2 | 1 | 165 |
| 470 | 1 | 2 | 1 | 99 |
| 447 | 5 | 1 | 1 | 185 |

number 466 was obtained from a cow that had aborted 17 days previously at 46 days of gestation. She was from the Garden City Experiment Station and no other history was available.

Six uterine samples, collected from slaughter house cows at the Armour Packing Company, had either dead embryos or embryonic membranes present in a degenerative condition (Table 5). Uterine samples from five cows that were an estimated 40 to 61 days post-partum were collected (Table 6). The breeding histories on these two groups of cows were not available or were limited.

Table 5. Samples from cows with dead fetuses or degenerating embryonic membranes.

| Sample No. | Estimated No. of days gestation | Remarks |
|---|---|---|
| 403 | 40 | No embryo present, only degenerating membranes. |
| 408 | 90 | Dead embryo, yellow exodate and degenerating placentome. |
| 409 | 35 | Dead embryo, pus in uterine horn. |
| 410 | 40 | Dead embryo, membranes unattached. |
| 414 | 60 | Only small particles of bone present. |
| 416 | 60 | Dead embryo. |

Table 6. Samples from post-partum cows.

| Sample No. | Remarks |
|---|---|
| 406 | 60 days post-partum, from K.S.U. dairy herd. |
| 421 | Estimated 40 days post-partum, from Kansas City slaughterhouse. |
| 422 | Estimated 50 days post-partum, from Kansas City slaughterhouse. |
| 423 | 57 days post-partum, from K.S.U. dairy herd. |
| 464 | 61 days post-partum, from Garden City Experiment Station herd. |

## Experimental Procedures

Chick Embryo Inoculation. The samples prepared from uterine washings and uterine mucosa were removed from storage and rapidly thawed. A $10^{-1}$ dilution was made by adding 0.5 ml of the sample to 4.5 ml of PBS containing 250 units of penicillin and 100 $\mu$g of streptomycin. The diluted samples were allowed to set in the refrigerator at 4° C for one to two hours to allow time for the bacteriostatic action of the antibiotics. The embryonated eggs were inoculated after this waiting period.

Yolk Sac Inoculation. Six eggs per sample were inoculated, harvested, and prepared for the subsequent serial passage as previously described. The eggs were incubated at 37° C for seven to nine days before harvesting. At the end of the incubation period the yolk sacs were harvested and yolk sacs smeared and stained with Giemsa's stain. Non-bacterial deaths or the presence of inclusion bodies in the stained smears were the criteria used to detect viral infections (Beveridge and Burnet, 1946).

Allantoic Cavity Inoculation. Six eggs per sample were inoculated, harvested, and prepared for the subsequent serial passage as previously described. The eggs were incubated seven to nine days at 37° C. The indications of viral infection were non-bacterial deaths, hemorrhages or edema of the embryos, retarded growth of the embryos, or abnormal allantoic fluids (Beveridge and Burnet, 1946).

Chorioallantoic Membrane Inoculation. Six eggs per sample were inoculated, harvested, and prepared for the subsequent serial passage as previously described. The eggs were incubated seven to nine days

at 37° C. Focal lesions on the chorioallantoic membranes were the primary signs looked for to detect viral infection. However, non-bacterial deaths, hemorrhages and edema of the embryos or chorio-allantoic membrane, thickening of the chorioallantoic membrane, or retarded growth of the embryo could also indicate a viral infection (Beveridge and Burnet, 1946).

Bovine Kidney Cell Cultures. Samples for inoculations of bovine kidney cell cultures were diluted and handled similarly to the samples for chick embryo inoculation. One-tenth ml of the sample was inoculated into the growth medium of the cell cultures. After inoculation, the cell cultures were handled by two different procedures. One procedure was to incubate the cultures for a period of 18 to 21 days, because beyond this time cultures began to degenerate because of their age. Uninoculated controls were used to distinguish degeneration because of age from degeneration due to viral infection. The cells were examined daily for cytopathogenic effects such as cellular degeneration, vacuolization of the cytoplasm, or death and lysis of the cells (Lynn and Morgan, 1954; and Madin, 1959). After the incubation period the cells were stained with Giemsa's stain, as previously described, and examined for degenerative changes of the cells.

The second procedure for handling of the cell cultures was to incubate the culture for one week. The cells were examined each day for cytopathogenic effects. At the end of the seven day incubation period the cultures were frozen and thawed three times. Ethyl alcohol and dry ice were used to freeze the cultures rapidly. This procedure served the purpose of disrupting the cells for release of any possible

cell associated virus. One-tenth ml of the growth medium was transferred into new cell cultures. Four serial passages were conducted in this manner with one week allowed for each passage.

## Experimental Results

Chick Embryo Inoculation. Samples 401-493 were serially passed in the yolk sac, allantoic cavity, and on the chorioallantoic for three passages. Infection with a virus was not detected in any of the samples. Samples 459-563, 466-469, 471 and 482 were serially passed in the allantoic cavity and on the chorioallantoic membrane an additional four passages. These samples were selected for further passages because they were "repeat breeders" or had histories of repeated breeding and were pregnant less than 35 days. No virus infection was detected in the additional four passages.

Bovine Kidney Cultures. Samples 401-485 were inoculated into cell cultures and incubated for three weeks. No cytopathogenic effects were detected during or at the termination of the experiments. Samples 459-463, 466-467, 471, 482, and 491-493 were serially passed in bovine kidney cell cultures for four passages. With the exception of samples 491-493 these samples were from "repeat breeder" cows or from cows with repeated breeding histories. No cytopathogenic effects were detected in any of these samples.

## DISCUSSION

All of the uterine samples cultured for the presence of viruses using chick embryo and bovine kidney cell culture techniques were

negative. Neither the changes in the yolk sac nor the changes of the chorioallantoic membrane that were reported by Millar (1955) were observed from any of the samples cultured. It would appear that the conditions causing the lowered fertility and embryonic mortalities in the cows in his study differed from the conditions present in cows included in this survey. The cows in Millar's study were described as exhibiting either a vaginitis or a genital discharge, whereas the cows in this study had never exhibited such signs.

Giroud et al. (1950) showed that an inapparent infection could cause a high incidence of embryonic mortality in rats. When Rickettsia prowazekii organisms were inoculated into pregnant rats from the sixth to eleventh day of gestation it was found that 40.9 per cent of the fetuses were absent at 20 days of gestation. Implantation sites were still evident in the uteri which indicated that fetuses had been present previously. The pregnant rats showed no signs of the infection, their temperatures remained normal and no other signs were observed. Recovery of the rickettsial organism from peritoneal fluid and demonstration of agglutinins in the blood stream were the only ways the infection could be detected. The rickettsial organisms could not be demonstrated in the reproductive tract.

If viruses are a contributing factor in the cause of embryonic death, they may manifest themselves in such a manner that would make their detection difficult. With newer techniques in cell culture methods being developed the detection of "latent" viral infections may be demonstrated more readily. Cells infected with latent viruses do not show their presence until they are unmasked by certain environmental

conditions or particular techniques (Madin, 1959). As previously discussed, a high incidence of bacterial infections of the uteri of low fertility cows was reported by many investigators, but a majority of these bacterial isolates were not considered to be extremely pathogenic. Madin (1959) states that the future may place more emphasis on mixed infections of viral and bacterial origin. It would seem that future work on lowered fertility from the virological aspect could be directed toward detection of either latent infections, inapparent infections, or mixed infections with viruses and bacteria.

## SUMMARY

Forty-eight uterine samples were cultured for presence of viruses using chick embryo and cell culture techniques. Most of the samples were taken from repeat breeding cows or pregnant cows with histories of repeated breeding. The yolk sac, allantoic cavity, and chorioallantoic membrane of the embryonated egg were inoculated and three to seven fluid passages were made. One to four passages in bovine kidney cell cultures were made and they were examined for cytopathogenic effect. No evidence of viral infections were detected by any of these methods.

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# VIROLOGICAL EXAMINATION OF UTERI FROM COWS WITH LOW FERTILITY USING EGG EMBRYO AND CELL CULTURE METHODS

by

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Economic losses due to infertility are of major concern to the dairyman and rank second only to low milk production as a criterion for elimination of cows from the dairy herd. Early death of the embryo, the cause of which is undetermined, accounts for a large percentage of these infertile cows. Extensive research in the fields of genetics, physiology, microbiology, and nutrition is being conducted in an effort to determine the causes of low fertility and embryonic mortality. In the field of microbiology the role of bacteria in lowered fertility has been studied with varying and controversial results. Viruses have been shown to cause untoward effects on the early developing embryo in many species of animals. It seemed plausible that a virus, or viruses, could produce similar effects on the bovine embryo in the early stages of development. In an attempt to ascertain if viruses could be associated with low fertility an attempt was made to isolate a filterable agent from the uteri of "repeat breeder" cows.

Over a period of nine months samples from the uteri of 47 cows were collected at the time of slaughter and cultured for the presence of a viral agent. The samples were cultured using the yolk sac, allantoic cavity, and chorioallantoic membrane of the embryonated egg and govine kidney cell cultures as methods for viral detection. A series of from three to seven blind passages in the embryonated egg and one to four passages in the cell cultures were carried out on each of the samples.

Using the chick embryo and cell culture techniques no viral agents were detected in any of the 47 samples cultured. It cannot be conclu-

sively stated that a virus is not associated with low fertility in dairy cattle from the results of this survey. The methods used in this study may be inadequate to detect certain viruses. Latent viruses may not manifest themselves unless the proper environmental conditions are present or special techniques are applied to unmask them.