INDUCTION AND DETECTION OF SPERM ANTIBODY IN THE BITCH

by

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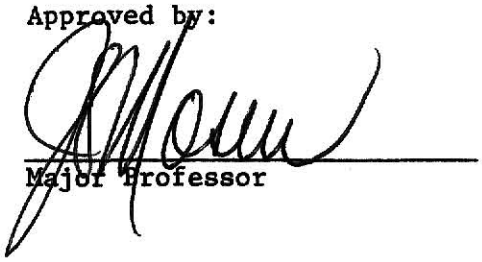

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INTRODUCTION

Canine reproduction has developed two important areas in recent years: production of puppies from planned breedings and the prevention of unwanted pregnancies. The role of the immune system in reproduction is not well understood and no information is available for the canine. Theoretically semen has the potential to produce antibodies systematically and in the reproductive tract of the bitch. These antibodies could be responsible for decreases in litter size or sterility. Antibodies to semen may eventually provide a mechanism for the prevention of pregnancy.

This study attempted to induce antibodies to semen in the bitch by using a method which simulated actual exposure.

REVIEW OF THE LITERATURE

Historic Review

Landsteiner (35, 1899) injected guinea pigs intraperitoneally with fresh bull semen several times. He then injected sperm intraperitoneally into this group and another group of guinea pigs which had not received prior injections. Peritoneal fluid and the sperm were immediately withdrawn. He observed that the sperm in the sensitized guinea pigs immediately lost motility whereas the sperm in the control guinea pigs did not.

McCartney (38, 1923) injected female rats subcutaneously with semen and noted an infertility for two to twenty-seven weeks. When these rats became pregnant there was a significant decrease in litter size. The estrous cycle remained unaffected.

Pommerenke (58, 1928) injected female rabbits intravenously with sperm and observed a toxic effect of vaginal secretions and serum on spermatozoa and a decrease in the life span of sperm normally deposited in the genital tract. Sperm reaching the uterus of the injected rabbits were nonmotile. Infertility lasted for a period of three to twenty-five weeks. The injections did not effect the estrus cycle.

Katsch (27, 1958) injected guinea pigs with guinea pig testis in saline subcutaneously or intraperitoneally or guinea pig testis and adjuvant intracutaneously and removed the uterine horns between the fifteenth and thirty-ninth days post injection. The tissue was tested for the response to homologous sperm. Results indicated a hypersensitivity to sperm which was similar in some respects but different from true anaphylaxis.

Katsch (29, 1959b) injected one group of female guinea pigs intracutaneously with a combination of Freund's adjuvant and either guinea pig,

rabbit, or bull sperm. Another group were injected with a combination of saline and either guinea pig, rabbit, or bull sperm. The degree of infertility was measured by the number of animals which littered and the length of time after exposure to a male. Only the guinea pigs injected with adjuvant plus guinea pig sperm showed a significant decrease in fertility. Two of the nine guinea pigs receiving guinea pig sperm plus adjuvant produced offspring compared to nine on nine of the controls.

McLaren (39, 1964) reported a decreased litter size and occasional infertility after sperm injections in mice. Three injections intracutaneously were given weekly for seven weeks after which agglutination titers were determined using the microagglutination technique. It was determined that normal matings and ovulation occurred in the immunized mice. The oviducts were examined for the presence of sperm and fertilized eggs. A low number of spermatozoa (.5 sperm in the oviduct) were recovered from the oviducts of the immunized animals in comparison to the controls (8.5 sperm in the oviduct). The number of fertilized eggs declined as the agglutination titer increased 1/2 the eggs were fertilized at titers of 1:512 whereas at titers of 1:1024 and greater no eggs were fertilized.

Moyer and Maruta (45, 1967) injected monkeys intramuscularly and subcutaneously with semen, washed spermatozoa, and seminal plasma and produced isoimmunization. The antibodies were detected using the passive hemagglutination test, microimmunodiffusion test, and spermagglutination test. Antibody was detected two weeks after the last injection and continued for thirty-five to forty-two days. In the sensitized monkeys a rise in circulating antibody was produced by placing soluble semen

protein intravaginally. The rise was of smaller magnitude and shorter duration than that which occurred with the injections. Phagocytosis of homologous sperm by uterine granulocytic leukocytes was seen using electron microscopic examination of uterine flushings in two of the sensitized monkeys.

Bell (1, 1969) injected rabbits in the foot pad, subcutaneously, and intravaginally with a sperm-alginate suspension weekly for ten weeks. The injected animals showed a significant decrease ($P = .026$) in pregnancy rates as compared to the controls. Ovulation occurred in seven of seven of the injected rabbits and nine of nine of the controls. The decreased pregnancy rate was apparently caused by failure of fertilization.

Burke, et al. (4, 1971) injected turkey hens with turkey semen plus Freund's adjuvant intraperitoneally. A depression ($P < .05$) was noted in fertility level. Antibodies to sperm when present in vaginal secretions either prevented the transport of spermatozoa into the storage glands or destroyed the spermatozoa or its fertilizing ability while present in the storage glands. Immunoglobulins detected in ovarian follicular fluid might possibly prevent fertilization or cause early embryonic death.

Omran (51, 1971) induced antibodies to sperm by inserting a tampon containing sperm into the cervix of cows. Tampons were left continuously in the cervix for five to six weeks and replaced twice weekly. Sera and cervical mucus were analyzed by microimmunodiffusion for sperm antibodies. The antibodies to sperm appeared first in the cervical mucus and six to ten weeks later appeared in the sera. The antibodies were present in the cervical mucus for twenty-two to twenty-six weeks while circulating antibodies appeared after six to ten weeks and remained only three to four weeks. These observations suggest a leakage of antibodies from cervical

mucus to blood. The six heifers with sperm antibodies had over twelve estrous cycles with matings without pregnancy.

Kiddy and Rollins (33, 1973) studied the fertilization and embryo survival rates in guinea pigs injected intradermally with guinea pig testis at fourteen day intervals. The fertilization rates were high in both the control (91.6%) and treated (88%) groups. The embryo survival rates showed a significant difference ($t = 2.19$, $P = .03$) between the control (72.7%) and treated (45.8%) groups. This seemed to indicate that infertility in these guinea pigs was due to early embryonic death. The cause of the early embryonic death was not determined however it is possible that implantation was interfered with.

Semen had been injected subcutaneously (1,27,29,38,39,45), intramuscularly (45), intradermally (1,33), intraperitoneally (4,27,35), intravenously (58), and intravaginally (1,45,51) into various animals with and without adjuvants and using various dosage schedules. Similar conclusions were made. In Landsteiner's original experiment loss of sperm motility was observed in peritoneal fluid (35) and later Pommerenke observed a loss of motility in the uterus (58). The injections of sperm caused no alteration in the estrous cycle (38,58,29,39,51) and ovulation occurred normally (1,39). The time interval from exposure to the male to delivery of young varied from two to twenty-seven weeks (28,38,39,51,58). A decrease in fertility (1,4,29,39) and decrease in litter size frequently occurred (38). Results suggest that either the spermatozoa failed to fertilize the ovum (1,33,39,45) or that early embryonic death occurred (33,51).

Antigenicity of Spermatozoa

Mudd and Mudd (48, 1948) showed that spermatozoa possess both species and tissue specificity.

Katsch (30, 1966) hypothesized that the reason females do not normally become sterile following repeated exposure to semen is because enzyme systems destroyed sperm antigens before exposure to the female immune system of the vaginal tract. Phagocytosis probably play a minor role.

Vickery and Bennett (70, 1968) discussed an antiagglutinic factor (AAF) found in cervical secretions of women, cows, rabbits, and sows which enhances spermigration. The production of AAF was depressed by progesterone and stimulated by estrogen indicating that the highest concentration of AAF occurred during ovulation. The ovarian follicles and in some species the Fallopian tubes produced AAF.

Roberts and Boettcher (60, 1969) reviewed the properties of sperm coating antigen (SCA). SCA originated from the seminal vesicle and was found in seminal plasma even in the absence of spermatozoa. It was the most antigenic component of seminal spermatozoa and was not detected on testicular or epidymal spermatozoa. SCA was a beta globulin of large molecular size.

Roberts and Boettcher (60, 1969) identified "SCA as an iron binding protein in seminal plasma, which was not identical with either transferrin or lactoferrin...termed 'scafferin'."

Defazio and Ketchel (8, 1971) determined that there were at least two components of cervical mucus which were immunologically identical to the nonserum components of seminal plasma. It is hypothesized that proteins of seminal plasma do not sensitize the female because the proteins of seminal

plasma also occurred in the secretions of the female reproductive tract. Other proteins may occur in concentrations too small for detection.

Menge (41, 1971) suggested that sperm isoantibodies prevented fertilization by inhibiting sperm cells from adhering to the ova in rabbits. Fluid from the oviducts contained an "antiadherent activity." Menge suggested that the ova was surrounded by antisperm antibodies entrapped in the cumulus mass which would inhibit adherence and penetration. The antibodies also covered the aniginic sites on the acrosomal membrane which are essential for spermatozoa ovum interaction producing the antiadherent effect.

Moghissi (45, 1972) reviewed the studies by P. E. Mattner who showed that after coitus live sperm follow the line of strain in the cervical mucus originating at the mucus secreting epithelium. Live spermatozoa are channeled to the mucus of the cervix rather than directly into the uterus. This results in an aggregation of sperm in the crypts and clefts of the cerival mucosa where they may be stored and slowly released at a constant rate into the uterus.

This mechanism tends to protect sperm from the leukocytes found in the central mass of the mucus. Dead sperm will not penetrate cervical mucus and tend to be confined to the central portion of the lumen.

Sperm migration can be impeded by a number of factors. The presence of cellular debri and leukocytes tend to impede progress. Extremely viscous samples of cervical mucus with high surface tension will initially impede sperm. Immotile sperm are unable to penetrate mucus. pH plays an important role in motility of sperm. The greatest activity of sperm occurs between the pH of seven to eight point five which occurs in normal mid-cycle

cervical mucus. Such factors are governed by the hormones of reproduction. Estrogen produces an acellular, thin watery, alkaline secretion which is receptive to sperm. Progesterone stimulates viscous cellular mucus which spermatozoa are unable to penetrate during the luteal phase.

Metz (44, 1973) reviewed his own and others work on the role of sperm antigens in fertilization. Sperm treated with antibody fragments did not result in agglutination or immobilization of sperm. Fragments called univalent antisperm antibody will combine with and block antigenic sites. Reduced conceptions occurred when univalent antisperm antibody treated sperm were introduced into the rabbit vagina. Uterine washings were performed and very few spermatozoa recovered indicating a blockage of the passage of sperm through cervical mucus.

The cervix was bypassed and the antisperm antibody pretreated semen was inseminated into the uterus; few conceptions were produced. It was thought there was an interference with capacitation. In normal rabbits sperm flushed from the uterus were attached to free leukocytes, however, this was not seen in rabbits inseminated with the univalent antisperm antibody. It is proposed that the leukocytes were essential to rabbit capacition.

Capacitated sperm treated with univalent antisperm antibody was placed in the oviducts of rabbits at the normal time for sperm-egg interaction. No cleaved eggs were recovered compared with 66% of the controls which showed early development. It was suggested that antigen were blocked which were required for sperm egg interaction.

The cumulus dispersing action of rabbit sperm and hyaluronic acid depolarimization had also been inhibited by antibodies. Hyaluronidase was shown to be immunologically species specific.

Mettler, et al. (43, 1974) noted that there two separate systems responsible for the antigenicity of sperm were: 1) Spermatozoa and testicular tissue components and 2) the secretions of the prostate and accessory glands. The antigens subdivided into three categories: sperm-specific antigens, blood group antigens and transplantation antigens. Theoretically, the latter two groups have the potential to cause infertility however conclusive evidence has not shown them to cause sterility.

Three major antigenic components have been isolated from semen. Spermatozoa were species and tissue specific (48) and contained specific antigens (43,45). The secretions of the prostate and accessory glands were shown to be antigenic (43). The most antigenic component of the seminal plasma is a large molecular beta-globulin (60) called sperm coating antigen or scafferin (60). It is an iron binding protein related to lactoferrin (60). Blood group antigens and transplantation antigens were shown to be present with no correlation between their presence and infertility (43).

It is suggested that females are not normally sensitized to these antigens because of a similarity between the proteins of a seminal plasma and the secretions of the female reproductive tract (8). The presence of enzyme systems which destroy the sperm antigens prior to exposure to the immune system had been hypothesized (30). Phagocytosis may have played a minor role (30).

Normally after coitus live spermatozoa appeared to follow the line of strain in cervical mucus and aggregated into the crypts and clefts of the cervical mucosa rather than entering directly into the uterus. The spermatozoa were released at a constant rate into the uterus. Dead sperm were confined to the central portion of the lumen (44). Cellular debri,

leukocytes and viscous cervical mucus impede progress and sperm had a chemotactic attraction for macrophages (25). Immotile spermatozoa are unable to penetrate mucus (45). Best motility occurs between the pH of seven to eight point five (45). Estrogen production produced an accelular, thin, watery, alkaline secretion in the reproductive tract which enhances sperm migration (45). At the time of ovulation an antiagglutinic factor was present in cervical secretion which enhanced sperm migration (70). Progesterone produces viscous cellular cervical mucus which spermatozoa are unable to penetrate (45).

Antibodies to sperm have been shown in rabbits to interfere with passage of sperm through the cervix and with capacitation (44). Antibodies to spermatozoa have been shown in fluid from these antibodies produced an "antiadherent" activity which inhibits sperm cells from adhering to the ova (44,41). It is suggested that the antibodies cover the antigenic sites on the acrosomal membrane which is essential for spermatozoal-ovum interaction (41). Antibodies were also present in the cumulus mean which inhibited adherence and penetration of the spermatozoa in to the area (43,44).

Cervicovaginal Secretory System

Govers and Girard (52, 1972) studied the concentration of IgG, IgM, and IgA in human cervicovaginal secretions. The IgG/IgA ratio was 1:5 and was present in only 26% of the samples. There was no significant difference in concentrations of immunoglobulins between non-infected patients, patients with a vaginal discharge, pregnant patients, and patients after menopause.

Pacheo-Romero et al. (53, 1973) found IgG more frequently in vaginal secretions than IgA. No difference in their presence was detected between the normal and infertile women.

Mettler et al. (43, 1974) studied the protein levels of cervical mucus of normal patients. The maximum amount of total protein peaked at the time of ovulation. The IgG concentration was also greatest during ovulation while IgA peaked at the end of the menstrual cycle and IgM was seldom detected. IgA was mainly detected in fertile women.

Jones (24, 1975) reviewed the immunologic competency of the female reproductive tract. The genital tract, particularly the uterus was able to mediate a local immune response. A large number of sperm had access to the genital tract and peritoneum. Sperm were phagocytized by somatic cells and possessed a chemotactic attraction for macrophages and neutrophils. Exaggerated humeral antibody response had been shown to semán in the presence of cervico vaginal infections.

Vaerman and Ferin (69, 1974) discussed the immune response of the cervix and vagina. The vagina appeared not to have a secretory immune system. There were no glands associated with the mucosa and the IgA and IgM plasma cells were absent from beneath the vaginal epithelium. No secretory component was found in the epithelium.

The cervix appeared capable of functioning as a local secretory immune system. As IgA and IgG plasma cells were noted subepithelially, and a secretory component was present in the glandular epithelium between epithelial cells and at their base. Both IgA and IgG were present in secretions at a ratio much higher than that of serum as would be expected in a secretory immune system.

Rebello (59, 1975) demonstrated that the endocervix has a local secretory system which produces primarily IgA. Type IIS IgA which is only produced

by a local secretory system comprises seventy per cent of the IgA in cervicovaginal secretions. The presence of type IIS also suggests that very little IgA comes from the serum.

The IgA IIS is linked to a glycoprotein called secretory component which protects the molecule form proteinolysis. This gives an advantage over serum IgA and IgG. These molecules when on the epithelial surfaces have an ability to neutralize viruses, exhibit antibacterial activity, activate complement, and form nonabsorbable stable complexes with exogenous antigens.

Coelingh-Bennink and Menge (5, 1974) reported IgM to leak in small amounts from the general circulation to the cervical mucus occasionally and considered it of biological importance in blood sera.

IgG in the cervical mucus was found to be one per cent of the blood concentration which was a 1:100 dilution factor in the cervical mucus. IgA sperm antibodies seemed to be produced locally with eventual leakage into the general circulation.

Ogra and Ogra (50, 1973) immunized women intravaginally and into the uterus with inactivated polio vaccine and studied the antibody response. The vagina predominantly produced IgA and to a smaller extent IgG immunoglobulin while the uterus predominantly produced IgG. Oral and intramuscular immunization of polio vaccine produced serum levels of IgG and IgA. Only IgG was detected in the genital tract during the time of highest IgG levels in the serum.

The vagina appears not to have a secretory immune system (69). The cervix is functional as an immune system (69) and along with the uterus produces the secretory immunoglobulins (24). The uterus produces primarily

IgG (50) and the cervix produces type IIS IgA and to a smaller extent IgG (59). Type IIS IgA with the secretory component comprising seventy per cent of IgA in the cervical secretions is produced by a local immune system and protects the molecule from protein lysis (59).

IgA appears to be produced locally and leaked systemically. Following systemic production of IgG and IgA to a specific antigen only IgG was detected in the genital tract. This occurred during the period following the highest IgG concentration in the serum (50). IgG in cervical mucus approximated one per cent of blood concentration (5). The 1:100 dilution indicated that only high blood titers were related to infertility (59). IgM was rarely detected (43,52) and appeared to leak into cervical mucus from the general circulation (5).

There is no agreement on the various concentrations of IgG and IgA in cervical vaginal secretions. When measured against a specific antigen (polio vaccine intravaginally) IgA is predominantly produced and IgG secondarily (50). Govers and Girard (52) state that the IgG:IgA ratio is 1:5. However Pacheo-Romero (53) noted that IgG is more frequently found than IgA. Two authors state there is no difference in the concentration of IgG and IgA between normal women, infertile women, pregnant women, women with a vaginal discharge and women after menopause (53,52). Mettler (43) states that IgA is mainly detected in infertile women. It is also stated that IgG concentration is greater after ovulation while IgA peaks at the end of the Menstrual cycle (43).

Microagglutination Test

Schwimmer et al. (64, 1967) studied sperm agglutination in infertile women using the microagglutination test. 45.3% of the women with primary

unexplained infertility, 53.21% of the women with unexplained secondary infertility and 34.1% of the pregnant women had sperm agglutinins compared with 20% of the controls.

Dukes et al. (10, 1968) measured the sperm-agglutinating capacity of women. Of the 487 patients studied 22.2% had positive agglutination. The patients were grouped into five categories and examined for agglutinating antibodies. The incidence of antibodies was as follows; women with primary infertility (67.2%), organic reasons for infertility (15.9%), known fertility (9.1%), unknown fertility (7.7%) and secondary infertility (20%).

Israelstam (22, 1969) reported the incidence of sperm agglutinating antibodies in 45 couples with infertility of no known cause. A slide test modification of the Franklin and Dukes test was used. Agglutination occurred in 29% of the cases however only 7% of the samples showed greater agglutination than the controls.

Robinovich (61, 1969) studied fourteen couples with infertility of unknown causes (Group A) and forty-two couples with infertility of known cause (group B) using the microagglutination test. 42.8% of group A and 19% of group B were positive.

Glass (14, 1970) studied 179 sterile couples for sperm agglutinating antibodies. Two of 46 patients (5%) were positive with organic causes for infertility. One hundred twenty-two patients had no evidence of organic illness and twenty-four (20%) were positive. Seventy-seven patients had primary infertility and thirteen (17%) of which were positive. Eleven (24%) of the forty-five couples with secondary infertility were positive.

Deo and Roy (9, 1971) studied the presence of sperm agglutination in the cervical mucus of repeat and normal breeding cows and buffalo cows. A

mild reaction was found generally in the normal and repeat breeding animals. Medium and high titer reaction occurred more frequently in the repeat breeding that in the normals. No correlation occurred between the agglutination reaction and pregnancy. There seemed to be evidence that there was probably insufficient antibodies present in cervical mucus to cause clumps of sperm due to agglutination.

El-Mahgoub (11, 1971) studied the relationship between infertility and cervical and/or vaginal schistomiasis. 63.4% of the infertile women had circulating serum sperm agglutinins as detected by the microagglutination test. 10% of the patients with urinary or gastrointestinal schistosomiasis were positive. It is not known if the infection of the cervix and/or vagina with Schistosoma causes production of antispermatozoal antibodies or if the antibodies produced a cross reaction.

Isojima et al (12, 1972) reported the microagglutination test had a positive reaction in 37.5% of the women with sterility of unexplained etiology and 45.8% of the pregnant women.

Pacheo-Romero et al. (53, 1973) found sperm agglutinating activity in 22% of the patients with primary infertility, theoretically not associated with detectable disease and 41% of the fertile patients had disease of the reproductive tract.

Mettler et al. (43, 1974) reported 7.5% of the one hundred fifty women had sperm agglutinating activity repeatedly detected. In forty six patients with primary unexplained fertility 17.4% had agglutinating sperm antibodies using the Franklin and Dukes microagglutination test.

Boettcher et al. (3, 1971) studied the nature of the agglutinins active in the sperm microagglutination test of Franklin and Dukes (SAT). The SAT

had agglutinins which were nonimmunoglobulins and had an electrophoretic mobility of beta globulins. This globulin was found among infertile women, pregnant women, and women taking oral contraceptives. IgG also occasionally agglutinated sperm in the SAT.

Grandl (16, 1975) purified spermagglutinating antibodies by affinity chromatography. Sera and cervical mucus from women with a positive Franklin and Dukes test were used. The sperm antibody fraction was determined by cross immunoelectrophoresis and the type of antibody was determined by absorption studies. The positive cases showed only one big peak which was IgM in the sera and IgA in the cervical mucus. No peaks occurred in the control sera or cervical mucus. Only a small number of cases were done so no conclusions could be made.

Dukes et al. (10, 1968) reported the sera of three patients with strong agglutinating antibody were absorbed with freeze dried pooled seminal plasma which did not significantly reduce the sperm agglutinating titer. When the sera was absorbed with spermatocile sperm there was a significant reduction in titers. This seemed to indicate that sperm agglutinating antibody which is measured by the Franklin and Dukes test is specific for antigens on the sperm and not for other components of the ejaculate. Dukes stated:

> "the presence of circulating sperm-agglutinating antibody in women is correlated with unexplained infertility. The circulating sperm-agglutinating antibody may be merely a reflection of the immunological process involved and not specifically responsible for the state of infertility."

Boettcher et al (2, 1970) showed the agglutinins in the sperm micro-agglutination technique appeared not to be antibodies. The spermagglutinins

studied were not IgG, IgM, IgA, or IgD and were not complement dependent. The sperm-agglutinating activity appeared to be caused by elevated levels of steroid transported by beta-lipoprotein which was the major transporter of sex steriods.

Hjort and Hansen (19, 1971) reported that antigens which caused fluorescence were not the same antigens involved in the sperm agglutination test.

Isojima et al (20, 1972) had a higher percentage of positive reactions with the sperm microagglutination test (Franklin and Dukes) among pregnant women than those cases of unexplained etiology. The presence of sperm agglutinins did not appear to correlate with sterility, however it is possible that it was the result of exposure to semen.

Parish et al. (54, 1968) reported on harmless antibodies which combined with the seminal plasma antigens on the surface of the spermatozoa. These antigens were the normal results of a response to foreign protein. There was no evidence that the antigens were cytotoxic or the cause of infertility. These possibly caused the agglutinating antibodies which were found in sera of fertile women.

Mettler and Grandl (42, 1975) studied the reproducibility of the Microagglutination test (Franklin and Dukes) and determined that it should not be used for quantification of the sperm agglutinating antibodies but only as comparison using the same semen sample on the same day. The per cent agglutination was more informative than agglutination titers.

Mettler (43, 1974) used the following criteria for a positive Franklin and Dukes sperm agglutination test:

> "a) ten out of one hundred motile cells are agglutinated, b) negative control system shows no agglutination, and c) a control test after four weeks reveals positive results."

A known positive control serum was also run.

Sperm agglutinating antibodies have been detected by the Franklin and Dukes test in women with primary, secondary, and unexplained infertility (10,11,14,22,43,53,61,64). Agglutinins have also been found in women who were fertile (10) and pregnant (64,21). No correlation was detected in cattle between the presence of agglutination and pregnancy (9).

Agglutinins measured by the Franklin and Dukes test are not well defined, Boettcher described them as a nonimmunoglobulins similar to a beta lipo-protein which serves as a transporter of sex steroids (2,3). Elevated levels of sex steroids produce elevated levels of beta protein and result in positive sperm agglutination test (2). IgG (3) and IgM (16) in sera and IgA in cervical mucus (16) have also caused agglutination which is not a complement dependent reaction (2). Fluorescent antibodies are not the antibodies involved in the microagglutination test (19).

The sperm agglutination test appears to measure antigens on the sperm and not other components of the ejaculate (10,54). This test is reproducible only as a comparison using the same semen sample on the same day (42).

The Macroagglutination Test and the Sperm Immobilization Test

Gupta et al. (17, 1975) studied seventy infertile couples and thirty fertile couples using the Kibrick method. In the infertile couples 5.7% of the infertile males and 10% of the infertile females were positive. None of the fertile couples were positive.

Isojima et al. (2, 1971) reported 34.5% of the women with sterility of unexplained causes and 37.5% of the pregnant women gave positive results when evaluated by the Kibrick technique.

Boettcher et al. (3, 1971) reported IgG and IgM type sperm antibodies to cause agglutination in the Kibrick technique. The Kibrick test appeared to be a more sensitive test than the Franklin and Dukes test when measuring the IgG antibody.

Mettler et al. (43, 1974) reported sperm immobilizing antibodies only in the sera of women with primary unexplained sterility using the test described by Isojima.

Isojima et al. (21, 1972) reported positive results in 17.6% of the women with sterility of unexplained etiology, 1% of the women with sterility of known cause and 0% of the controls using the sperm immobilizing test.

Isojima et al. (20, 1968) reported that sperm immobilizing behaved in an antibody-like behavior, fractioned in the gamma globulin portion, and depended on complement. Isojima et al. (21, 1972) reported the sperm immobilizing agent was mainly the seven S gamma globulin fraction in serum.

Isojima et al. (20, 1968) reported that the sperm immobilizing factor was an antibody against plasma-specific antigens and possibly against spermatozoa.

Jones and Ing (25, 1974) reported that seven of eight sera which showed agglutinating and immobilizing activity against spermatozoa had IgG antibodies binding to the tail and three also had IgG present on the acrosome. Four of twenty-two sera contained an agglutinated spermatozoa with antibody binding to various parts of the sperm. This suggests a correlation between complement dependent sperm immobilization and IgG binding of the tail. IgM shows some association between acrosomal staining and sperm microagglutination and sperm immobilization activity.

Petrunia et al. (56, 1976) noted that sera binding anti-IgM were largely from patients who had not demonstrated antispermatozoal activity with sperm microagglutination or immobilization.

Parish et al. (54, 1968) reported three couples who were normal except inactive sperm were found after coitus. Two different cytotoxic antibodies were detected and three harmless antibodies were detected. The cytotoxic antibodies were directed against antigens intrinsic to the cell while harmless antibodies reacted with proteins coating the cell which were acquired from the seminal plasma. One cytotoxic antibody was an IgG complement dependent antibody which immobilized the spermatozoa and caused deformation of the head. This antibody was found in the patients cervical mucus and serum. The other cytotoxic antibody was IgG but not complement dependent; it only caused immobilization of the spermatozoa. It was not neutralized by cell free seminal plasma and was present in the cervical mucus.

The Kibrick test for measuring macroagglutination has been used on the sera of pregnant (2), fertile (17), and infertile (17,21) women. IgG and IgM antibodies are responsible for the reaction (3).

Sperm immobilizing antibodies which were complement dependent have been only reported in sera of women with unexplained sterility (21,43) or sterility of known cause (21). The seven S gamma globulin fraction of serum (7,20) and IgG with (20) and without (54) complement are responsible for the immobilization. The IgG acts on the head or tail of spermatozoa (9). IgM has some association in sera with sperm microagglutination and immobilization and the acrosome (8,56). The immobilization antibody appeared to be specific against the seminal plasma antigens and spermatozoa (20,54).

Immunofluorescence of Sperm

Hjort and Hansen (19, 1971) reported fluorescence in fixed spermatozoa using normal sera at various dilutions. The acrosome reacted with a bright fluorescence covering the anterior two thirds of the head with a distinct, straight posterior limitation or a crescent shaped fluorescence. The equatorial segment was often combined with staining of the acrosome but also fluoresced by itself. Such results seemed to indicate two or three antigens in the acrosome: one in the front part, one at the equator, and possibly a common antigen to both. The postnuclear region produces two strongly fluorescent bands with weakly staining area between. The front band begins just behind the equatorial segment. This region produced the most infrequent pattern. The tail stains except for the upper part next to the neck whereas when nonspecific tail staining occurred the entire tail was involved. It was thought that some of the weak tail staining seen in the sera of children may be a cross reaction with lactoferrin, a common antigen in milk and semen.

Scarselli et al. (63, 1973) reported that in twenty of eight-six sterile women with a positive immunoflourescence test 75% had antibodies localized in the acrosome, 50% in the post acrosome, 30% in the tail and 10% in the equatorial segment. Pregnant women who were positive had flourescence in a single location. Two cases occurred in the acrosomal area and two cases occurred in the post acrosomal area.

They found that titers of less than 1:20 tended to arrange themselves in single locations while titers greater than 1:20 almost always occurred in various areas of the sperm.

Coelingh-Bennink and Menge (5, 1974) showed three out of thirteen women with unexplained fertility had spermatozoal antibodies in cervical mucus as detected by indirect immunofluorescence. One patient had IgG antibody against the main tail piece, another had an IgA antibody against the post nuclear cap and the third had IgA antibody against the main tail piece.

Hjort and Hansen (19, 1971) reported eight sera which reacted to IgM and six sera which react to IgG from infertile women. IgA was found in three cases by using the three layered immunoflourescent test. The front part of the acrosome reacted to primarily IgM and the tail reacted exclusively or predominantly with IgG class antibodies.

Jone and Ing (25, 1974) reported localization of anti-IgG and anti-IgM conjugates from thirty-two infertile women on one or more of five areas of sperm: whole acrosome, equatorial segment, neck, midpiece, and tail. No positive results were seen with anti-IgA conjugates. Staining of the equatorial and neck regions were thought to be nonspecific since it was not blocked with unconjugated anti-serum. Staining of the midpiece was rare and its specificity could not be determined.

Nine samples in which the acrosome stained had two flouresence with IgG; six with IgM and one with IgG plus IgM. IgG caused flourescence if the tail in all nine of nine cases.

Petrunia et al. (56, 1976) suggested that the large number of positive reactions which occurred using the methanol fixed spermatozoa was due to an alteration in the surface membrane. It was suggested that this alteration was caused by the methanol and allowed nonspecific binding of immunoglobulin and/or fluoresein-labelled anti-human immunoglobulin antibodies.

Hjort and Hansen (19, 1971) report that eighty to ninety percent of apparently normal spermatozoa will reveal characteristic flourescence. There does not appear to be any individual specificity of the antigens however organ specificity does occur. Serum that is undiluted or diluted 1:4 showed reactions in normal and infertile women which are probably of little significance. At high titers infertile women only had fluorescence of the acrosome, equatorial segment or the tail.

Fluorescence occurred on the acrosome, equatorial segment, postnuclear region and the tail (19). At undiluted and low titers this was not a significant finding since it occurred in normal and infertile sera (19). At higher titers (greater than 1:16) fluorescence occurred mainly in infertile women (19). At titers less than 1:20 fluorescence occured in single locations, at higher titers fluorescence occured at multiple sites (63).

The predominant antibodies in sera of infertile women were IgG and IgM (19). The acrosome reacted with primarily IgM (19,25) and to a lesser degree IgG (25). The tail stained with IgG conjugates (19,25) and IgA appeared only occasionally in the sera (19,25). IgA was the predominant immunoglobulin which fluoreces in cervical mucus of infertile women (5). It appeared against the postnuclear cap in one patient and the main tail piece in another (5). IgG conjugates were seen against the main tail piece (5). From only eighty to ninety per cent of normal spermatozoa revealed fluorescence with this technique (19). There was no individual specificity however organ specificity occurred (19). Petrunia (56) suggested that methanol caused an alteration in the surface membrane which resulted in a nonspecific binding of the immunoglobulins. The large number of positive reactions were explained by this theory.

Comparison of Tests

Isojima et al. (20, 1968) compared the sperm agglutination test (SAT), and the tanned sheep red blood cell agglutination test (BCAT), and the sperm immobilization test (SIT). The BCAT was a sensitive method but did not detect the substance (antibody) which causes the death of sperm. The SAT was difficult to interpret because spontaneous agglutination occurred. The SIT appeared to be more satisfactory.

Davajan et al. (7, 1972) discussed the significance of sperm agglutinating and immobilizing antibodies in women. Sperm agglutinating antibodies have not been detected in cervical mucus even if they are present in the serum. However sperm immobilizing antibodies have been detected in a small number of infertile couples. It was the only clinical sign which indicated an immune aspect of infertility.

Hansen (18, 1974) compared the gelatin agglutination test by the Kibrick method, microagglutination test by the Franklin-Dukes method, and sperm immobilization test by the Isojima method using the sera of infertile men and women. The results were divided into two categories. The first was sera with a positive reaction to the Kibrick test. High concentrations of agglutinating antibody in this sera also immobilized sperm. The second category was composed of sera which was positive using the sperm agglutination test. All sera which produced head to head agglutination in the sperm agglutination test was negative in the gelatin agglutination test and no sperm immobilization occurred. Sera which produced tail to tail agglutination was positive in the gelatin agglutination test also. Low titer sera in the Kibrick test was sometimes positive in the sperm agglutination test however high titer sera was negative.

Jones et al (26, 1973) used the sperm microagglutination test and the sperm immobilization test to evaluate infertility in women. There was no significant differences noted between women with organic causes, and unexplained causes of infertility and pregnant women using the micro-agglutination test. The sperm immobilization test had no positives among the controls (pregnant women) indicating a reliable means for detecting circulating antisperm antibodies which may be clinically significant. It was noted that several cases of unexplained infertility with strongly positive microagglutination also had high sperm immobilization values.

Petrunia et al. (56, 1976) compared sperm microagglutination and sperm immobilization using women with infertility due to various causes and pregnant women as the control. Statistically there was no significant difference between the groups using the sperm agglutination test. Sperm immobilization was significantly higher in women with unexplained fertility than any other group or pregnant women.

Pacheo-Romero et al. (53, 1973) showed that the presence of sperm agglutinating activity had little relationship to the ability to conceive. 50% of the women with sperm agglutinating antibody conceived and 38% of the women without conceived. Agglutinin activity of vaginal secretions was only found in 10% of all the patients, all of whom had activity in their serum.

Jones et al. (25, 1974) thought that the clinical significance of the microagglutination and sperm immobilization tests were restricted to patients with long standing infertility of undetermined origin.

Soffer et al. (68, 1976) studied the sperm immobilization test and the spermatotoxicity test on cervical mucus and sera of twenty-four women with primary or secondary sterility. No correlation was noted between the tests.

The sperm immobilizing test provides the most consistent indicator of infertility due to immune causes (7,20,26,56). The gelatin agglutination test (Kibrick test) and the microagglutination test (Franklin and Dukes test) only appeared to correlate with infertility at high titers (greater than 1:16) (18,26). The clinical significance of spermaggutinating and sperm immobilizing antibodies is restricted to long standing infertility (25). The ability to conceive appeared to have little relationship to the spermagglutinating activity (53).

Incidence of Abortion

Schwimmer et al. (64, 1967) saw no increase in the incidence of clinical abortion in women with sperm agglutinins.

Robinovich (61, 1969) observed that the women with infertility of unknown origin who had a positive sperm agglutination test showed a significant history of abortion during the first trimenster.

Jones et al. (25, 1974) noted that there was a higher incidence of spontaneous abortion among patients with sperm antigens.

Early abortion has been reported by two authors (25,61) and a third (64) recorded no increase in patients with sperm agglutinins.

Therapy

Mettler et al. (43, 1974) reported avoidance of sperm contact for long periods of time as the only known effective therapy. Cortisone does not appear to be efficatious and desensitization or immunosuppressive treatment are only speculative.

Glass (14, 1970) reported that nine of twenty-four (38%) women with positive sperm agglutination tests became pregnant. All of the women who became pregnant used condom therapy for three to six months.

The only reported effective therapy was avoidance of contact with sperm (14,43).

Vaginal Cultures

Osbaldistan et al. (52, 1972) reported the following isolates from vaginal cultures of fertile beagles: Hemophilus sp., Pseudomonas, Corynebacterium, Escherchia coli, enterococcus, Staphylococcus, Streptococcus, Bacterioides melanogenians, Hemophilus aphrophilus, and Bacteroides sp.

Moreno et al. (46, 1973) isolated the several organisms from the vaginal cultures of clinically normal bitches: Staphylococcus aurus (23.8%), Eschenichia coli (22.5%), Beta-hemolytic streptococi (20.5%) Psuedomonas aeruginosa (15.2%), Proteus mirabilis (8.6%), Candida albicans (5%), Proteus morganii (2.9%), and Aerobacter aerogenes (1.5%).

Platt and Simpson (57, 1974) isolated the following organisms from the vaginal tract of normal bitches: Staphyloccus epidermis (23.5%), Bacillus spp. (14.9%) Escherichia coli (10.4%), Proteus spp. (10.4%), Klebsiella-aerobacter (8%), Corynebacterium spp., Staphylococcus aureus (6.9%) beta-hemolytic streptococcus (6.4%), Pseudomonas aeruginosa (4.2%), citrobacter (4%), Pasteurella spp. (2.1%), Corynebacterium pyogenes (1.8%).

Only a limited number of studies of normal vaginal microflora of the bitch have been reported (46,52,57). These studies have used limited types of culture media with very few selective medias. The bitches cultured had either no history of reproductive tract infections (46,57) or were fertile beagles (52) and all samples were taken without regard to the phase of the estrus (46,52,57) cycle.

MATERIALS AND METHODS

Fifteen beagle bitches between the ages of five months and six and three fourths months were obtained from a commercial source. The dogs were housed in open air runs with access to shelter. Dry commercial dog food* and water were available ad libidum. Each dog was given a routine physical examination and a vaginal culture was taken. The bitches were randomly divided into three groups.

Group One received a submucosal injection of a mixture of .5cc of 6.8pH saline, .1cc of killed Escherichia coli, and .1cc of killed streptococcus. Group Two was injected with .5cc of 50,000 spermatozoa per cc and Group Three received .5cc of 50,000 spermatozoa per cc, .1cc of killed streptococcus, and .1cc of killed Escherchia coli. All injections were made into the left lateral vaginal wall. The injections were repeated fifteen days later. 4cc of blood was collected at the time of the first injection.

All animals were observed frequently for signs of estrous. Swelling of the vulva and bloody discharge were the signs used to detect estrous. The first day of proestrus occurred when the vulva was swollen three times normal size.

On day five of the estrous cycle a vaginal culture was obtained. Vaginal smears were made on day five, seven, nine, ten, and twelve. Cervical secretion washings were taken on days ten and twelve.

Preparation of Semen: Semen initially was obtained from an adult German Shepherd by manual manipulation of the penis without the presence of a bitch. A warmed sixty cc syringe casing was used for collection.

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*Purina

The semen was examined for morphology, motility, the presence of debri and counted (6). Appropriate dilutions were made using isotonic saline. The German Shepherd provided the semen for the sperm injections in the bitches and the rabbits. Semen to perform the laboratory tests was obtained from a Laborador Retriever and a mixbreed in the manner described for the German Shepherd. Semen was used within one hour of collection. Immediately after collection it was allowed to sit at 37°C to allow nonmotile and dead spermatozoa and debri to sedimen. The supernatant was removed, counted, and adjusted to forty to fifty million spermatozoa per cc.

Culture Techniques: A sterile glass speculum was inserted in the posterior vaginal vault and a sterile cotton swab was inserted to the area of the cervix. The swabs were placed in a sterile container and immediately transported to the laboratory.

The swabs were placed in one milliliter of tryptic soy broth and swirled for ten to fifteen seconds on Vortex Mixer. Fluid was expressed from the swab by pressing against the inside of the tube and a plate was inoculated and the swab was remoistened each time until all the plates were swabbed. Two blood agar (bovine blood), two phenylethyl-alcohol (PEA), one MacConkey, one Pseudocel, one Enterococcal Agar (EC), and one LBS with tomato juice were inoculated with each specimen. One blood agar, one PEA, the MacConkey and Pseudocel were incubated in CO_2 (5%) at 37°C. The other blood agar, PEA, the EC and LBS were incubated anaerobically in a BBL GasPak at 37°C.

The plates were checked at 24 hours and notation made as to colonies which were present. At 48 hours attempts were made to isolate each

type of colony present on the plate. The isolate specimens were inoculated in blood agar and returned to the original incubation conditions.

Organisms were identified using the Bergy's Manual, CDC's identification scheme and the Manual of Clinical Microbiology (Second Edition).

Vaginal Smears: The vaginal smears were obtained via a glass speculum and inserted into the posterior of the vagina. Water served as the lubricant. A sterile cotton swab moistened with saline was inserted into the vagina and rotated on the anterior vaginal walls. The swab was immediately rolled on a slide and immersed into ninety-five percent ethanol. All vaginal smears were stained using the Sano Trichrome method (62). Eight smears were stained with Wright's stain.

For evaluation the slide was divided into three sections and a total of one hundred cells were counted divided between each section. Red blood cells, white blood cells, cornified and noncornified cells were recorded. A noncornified cell was defined as a cell with a prominent nucleus with rounded or slightly flattened cytoplasm. The criteria for a cornified cell was a nuclear cell or a cell with a pyknotic nucleus and flattened edges to the cytoplasm. The slide was also evaluated for debri recording 1+ for small amounts aof 4+ for large amounts.

Preparation of Serum: All blood samples were obtained from the jugular vein. The blood was allowed to clot, the serum removed and frozen until processed. The frozen serum was thawed at room temperature and then heated to 50°C for one half hour to destroy the complement. All samples were serially diluted with isotonic saline.

Preparation of Cervical Washings: Cervical washings were collected on day ten and twelve of the estrous cycle. The bitches were anesthetized

with a short acting barbituate anesthesia*, the glass speculum inserted into the posterior one third of the vagina, and the caudal half of the bitch slightly elevated. One and one half to two cc of isotonic saline was infused into the vagina and immediately aspirated with a Pasteur pipette. The washings were frozen until required, then thawed at room temperature. The samples were heated at 50°C for 30 minutes to destroy the complement. All washings were tested undiluted and at a 1:4 dilution; isontonic saline was used as the diluent.

Preparation of the Killed Bacteria: Vaginal swabs were taken from two clinically normal bitches, cultured, and a species of streptococcus and Escherichia coli were isolated. The organisms were inoculated into Todd Hewett broth and incubated for twenty-four hours. The number of organisms present in the broth were determined by serial dilutions into tryptic soy agar pour plates. The bacteria were killed by placing in boiling water for one hour. The killed bacteria were plated on blood agar to demonstrate a 100% kill.

Preparation of a Known Positive: Two adult rabbits were injected subcutaneously in four to five locations using a one to one mixture of sperm and Freund's complete adjuvant. One rabbit received 1,000,000 spermatozoa and the other received 2,000,000. Sixteen days later the injections were repeated using 2.6 million spermatozoa mixed with an equal part of Freund's complete adjuvant. One month later the rabbits were bled using the lateral ear vein. The blood was allowed to clot, the serum removed and heated to 50°C for one half hour.

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*Surital, Parke-Davis

The Microagglutination Test: The microagglutination test (12) was performed on all sera and cervical mucus. .05 ml of a sperm suspension containing forty to fifty million spermatozoa per cc was added to 1.5 ml of the bitch's cervical mucus or sera. The serum was diluted serially from 1:4 through 1:64 and the cervical mucus was used undiluted and at a 1:4 dilution. The samples were incubated at 37°C for 60 minutes. At 30 minutes and 60 minutes small aliquots were examined for evidence of agglutination. A positive, negative, and saline control samples were also run.

Agglutination was defined as the aggregation of two or more motile sperm per high powered field. Agglutination of nonmotile sperm or adhesion of sperm to other cells was not included. The slides were examined for the degree of agglutination: 1+ -0 -1 agglutinated sperm per high powered field; 2+ -2 -4 agglutinated sperm per high powered field; 3+ 4 -6 agglutinated sperm per high powered field; 4+ 6 - 10 agglutinated sperm per high powered field. The type of agglutination was also evaluated: head to head, tail to tail, and mixed (26).

The Macroagglutination Test: The macroagglutination test (32) (gelatin aggluntination, Kibrick test) was performed on all serum samples. The gelatin was prepared with isotonic saline in a 10% solution. The gelatin solution was mixed with equal volumes of spermatozoa adjusted to 20 to 30 million per cc. This was maintained at 37°C to facilitate pipetting.

Serial dilutions of the serum from 1:4 through 1:64 were made using isotonic saline and .2cc were pipetted into a 50 x 6 mm tube. .2cc of the sperm gelatin mixture was added and mixed by inversion. The tubes were incubated for 90 minutes at 37°C, refrigerated, and read after solidifying. Positive, negative, and saline controls were also run.

If beginning sedimentation or nonspecific agglutination was observed in the control samples the test was terminated and final readings were taken. The tubes were graded, the size of the particles were also recorded: 1+ very small; 2+ medium sized; and 3+ large sized (32).

The Sperm Immobilization Test: The sperm immobilization test (20) was conducted on samples of cervical mucus. An undiluted and a simple 1:4 dilution of the cervical mucus were run. .25ml of the sera of cervical mucus was added to .05ml of a sperm solution standardized to 40 - 50 million spermatozoa per cc and .05 of complement. The samples were incubated at 37°C for 60 minutes. A saline control was run and each sample of the cervical mucus was run without adding complement.

100 cells were counted and the percentage of immotile cells calculated for both the normal and the test sample. The percentage of immotile spermatozoa from the sample without complement was divided by the percentage of immotile spermatozoa in the text sample to determine the sperm immobilization value (SIV).

RESULTS

Preparation of Semen

All semen samples used had sperm counts greater than fifty million spermatozoa per cc and had a minimum of 60% motile spermatozoa. Initial dilutions for the injections of semen were made with phosphate buffered saline. The dilutions for all the tests were made using isotonic saline since phosphate buffered saline caused autoagglutination.

Vaginal Cultures

The results of the vaginal cultures are listed in the appendix in Table 13 and 14. From 29 cultures 56 organisms were isolated in the anestrus cultures and 33 organisms from the proestrus sample.

The results of the vaginal smears are listed in the appendix in Tables 15 through 17. The staining of the slides was very poor and it was difficult to properly identify the cell types. The poor staining quality could be the result of a technician error or faculty stain.

Sperm Microagglutionation Test of Sperm

The results of the sperm microagglutination tests of serum are listed in Tables 1 through 3. Head to head agglutination was the predominant form of agglutination (Figures 1-4) although occasionally mixed agglutination or even more rarely tail to tail agglutination would occur. Motile sperm agglutinated to dead spermatozoa or debri and masses of dead sperm were considered nonspecific agglutination and occurred frequently in all samples (Figures 3 and 4).

Each animal served as its own control since the A sample was collected at the time of the first intravaginal injection of semen. The B sample

was collected on day ten of the estrous cycle. All the bitches except 705 (A), 709 (A) and 712 (A) showed sperm agglutination in the serum at the 1:4 dilution. Three of the five bitches (60%) of group one also showed agglutination at the 1:8 dilution. Of these three only one, 702 (B), showed an agglutination titer at 1:16 and 1:32.

All five bitches in group two showed agglutination at 1:16 although bitch 714 showed 1:16 agglutination in the A sample and none in the B sample. The only bitch to show agglutination greater than 1:16 was 699 (B) who was positive at 1:32. Four of the five bitches showed an increase in agglutination titers between the initial sample (A) and the estrous sample (B). Larger numbers of agglutinated sperm were present in the samples of two bitches in this group (699, 700).

Bitch 705 had no visible sign of estrous during the time of the study and was not included. Three of the four remaining bitches showed agglutination at 1:8. However only bitch 701 showed agglutination at 1:16. This is also the only bitch in this group whose estrous sample which agglutinated spermatozoa was present in larger numbers than the preinjection sample.

Sperm Microagglutination Test of Cervical Mucus

The results of the sperm microagglutination of cervical mucus test are listed in Tables 4 through 6. The A sample was taken on day 10 of the estrous cycle and the B sample on day 12.

In group one only bitch 708 (B) produced positive sperm agglutinating antibodies. None of the bitches in group two produced sperm agglutinating antibodies in the cervical mucus. Four of the five bitches in group three appeared to produce a lethal factor in the undiluted cervical mucus;

this factor also occurred in bitch 701 at the 1:4 dilution. Only bitch 708 (B) produced positive sperm agglutinating antibodies.

Macroagglutination Test of Serum

The results of the sperm macroagglutination test of serum are listed on tables 7 through 9. Sample A was obtained prior to the first injection and sample B was obtained on the 10th day as estrous. The numbers indicated the size of the particles observed as described in the Materials and Methods. The size of the particles were consistent in each sample, i.e., no mixed reactions occurred. The clarity of the gelatin media was directly related to the particle size - the larger the particle; the clearer the dispersing media. A one plus reaction indicated very little or no agglutination of sperm occurred. A two plus reaction showed the occurrance of some agglutination of sperm into small particles. The large particles which were present in the three plus reaction was caused by large aggregations of agglutinated sperm.

All five bitches in group one had a two plus or greater agglutination at a 1:64 dilution in the estrous sample. Three of the five bitches (702, 703, 713) had two plus or greater agglutination at the 1:64 dilution in the initial samples. Group two showed very little variation in agglutination between the initial samples and the estrous samples. Bitch 710 was the only animal to increase the amount of agglutination between sample A and B. The other bitches maintained the same degree of agglutination between samples: 699, 700, 709 - one plus and 714 - two plus, in the 1:32 and 1:64 dilutions. Two bitches (701, 704) in group three increased the degree of agglutination in the 1:32 and 1:64 dilution from one plus to two plus. The other two either remained the same or decreased the degree of

agglutination (712). 706 showed an increase in the degree of agglutination at the 1:32 dilution: one plus to three plus.

Sperm Immobilization Test

The sperm immobilization test was performed on the cervical mucus in a 1:4 dilution of two randomly selected bitches from each group. The results are listed on Tables 10 through 12. The complement appeared to have direct spermatotoxicity. The saline and complement control showed a 99% nonmotile spermatozoa compared to 32% nonmotile spermatozoa in the saline control. There was no difference noted between the bitches or the groups because of the toxicity of the complement.

Figure one to four show positive sperm agglutination. Figure three (on the right hand side) shows nonspecific agglutination.

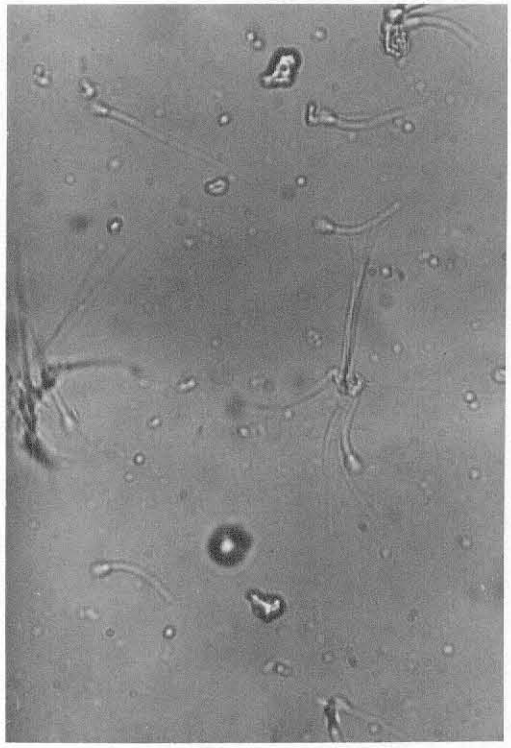

Figure 2

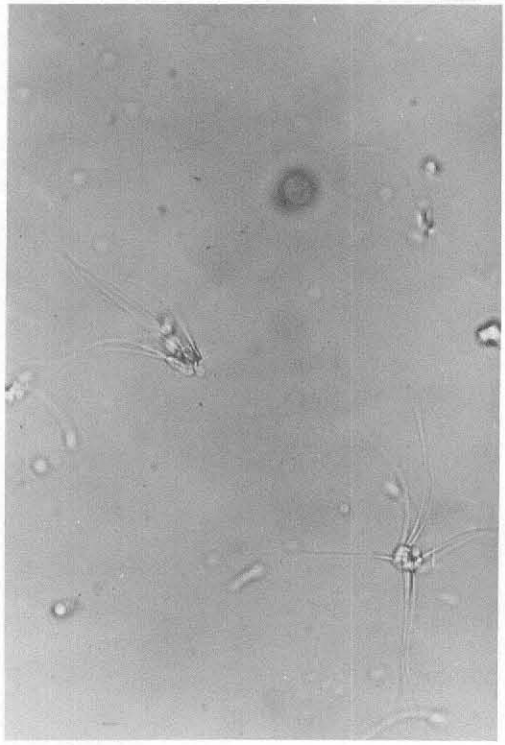

Figure 4

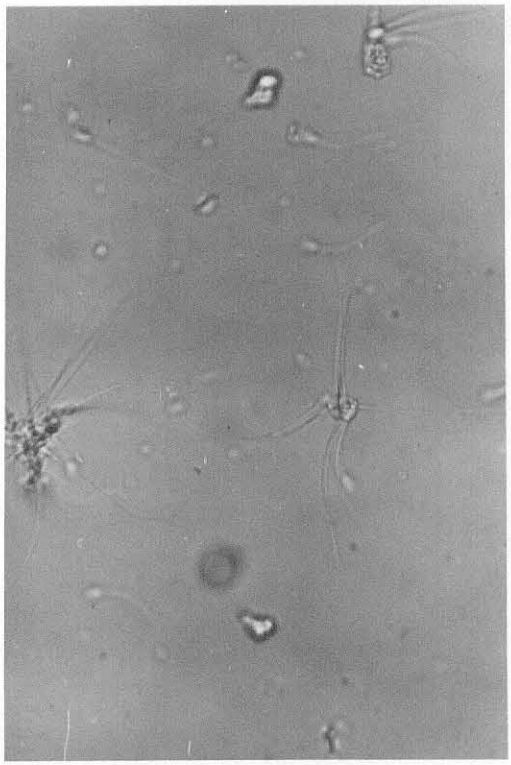

Figure 1

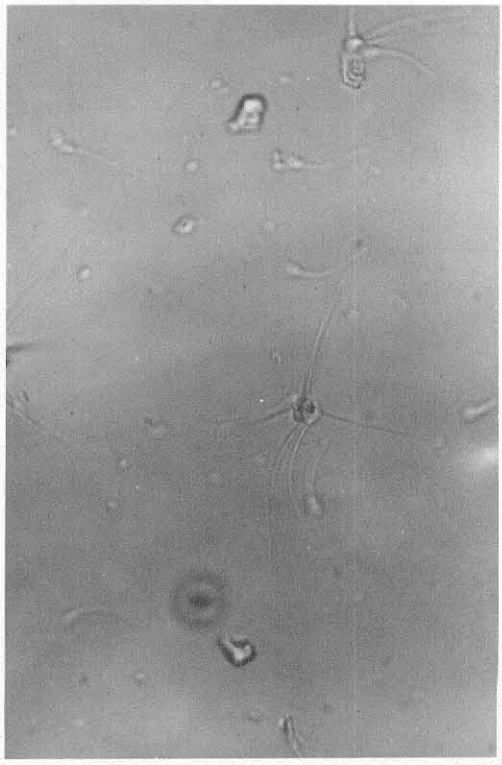

Figure 3

Table 1

Sperm Microagglutinating Antibody in Serum

		Group 1				
		1:4	1:8	1:16	1:32	1:64
702A	30 min	+2	+2	-	-	-
	60 min	-	-		-	-
702B	30 min	+	+	-	-	N
	60 min	+	+	+	+	-
703A	30 min	+	-	-	-	-
	60 min	+	-	-	-	-
703B	30 min	+	-	N	-	-
	60 min	+	-	N	N	N
708A	30 min	-	-	-	-	-
	60 min	+	-	-	N	D
708B	30 min	N	N	D	D	D
	60 min	+	+	-	D	D
711A	30 min	+	-	-	-	-
	60 min	+	+	-	-	-
711B	30 min	+	+	-	-	-
	60 min	+	N	N	N	N
713A	30 min	+	-	-	-	-
	60 min	+	-	-	-	-
713B	30 min	-	-	N	-	-
	60 min	+	-	-	N	D

N - all spermatozoa nonmotile
D - all spermatozoa dead

Table 2

Sperm Microagglutinating Antibody in Serum

		Group 2				
		1:4	1:8	1:16	1:32	1:64
699A	30 min	1+	-	-	-	-
	60 min	2+	-	-	N	N
699B	30 min	3+	1+	2+	1+	-
	60 min	3+	1+	-	-	-
700A	30 min	1+	1+	1+	-	-
	60 min	-	1+	-	-	-
700B	30 min	2+	2+	1+	-	-
	60 min	2+	2+	1+	N	-
709A	30 min	-	-	-	D	D
	60 min	-	1+	-	-	N
709B	30 min	1+	N	N	-	-
	60 min	1+	1+	-	-	D
710A	30 min	1+	-	-	-	-
	60 min	-	-	-	-	-
710B	30 min	-	+	-	-	-
	60 min	+	+	-	-	-
714A	30 min	-	-	+	D	N
	60 min	+	+	+	D	D
714B	30 min	-	-	-	-	-
	60 min	1+	1+	-	-	-

Table 3

Sperm Microagglutinating Antibody in Serum

		Group 3				
		1:4	1:8	1:16	1:32	1:64
701A	30 min	+	-	-	N	-
	60 min	+	-	-	N	N
701B	30 min	-	-	-	-	+
	60 min	+3	+2	+1	-	-
704A	30 min	+1	-	-	D	-
	60 min	+2	+1	-	-	-
704B	30 min	+2	+1	-	-	-
	60 min	+2	-	-	-	-
705A	30 min	-	-	-	-	-
	60 min	N	-	N	D	D
706A	30 min	+1	-	-	-	-
	60 min	+1	+1	-	N	N
706B	30 min	+2	+1	-	-	-
	60 min	+1	+1	-	-	-
712A	30 min	-	-	-	-	-
	60 min	-	-	-	D	-
712B	30 min	-1	-	-	-	D
	60 min	-	-	-	-	-

Table 4

Sperm Microagglutinating Antibodies of Cervical Mucus

Group 1

		undiluted	1:4
702A	30 min	D	D
	60 min	-	-
702B	30 min	+1	+1
	60 min	D	-
703A	30 min	-	QNS
	60 min	D	QNS
703B	30 min	-	-
	60 min	-	-
708A	30 min	D	-
	60 min	D	D
708B	30 min	+3	-
	60 min	+1	-
711A	30 min	-	-
	60 min	+1	-
713A	30 min	D	-
	60 min	D	-
713B	30 min	-	-
	60 min	N	D

D - all spermatozoa dead
M - all spermatozoa nonmotile
QNS - quantity not sufficient

Table 5

Sperm Microagglutinating Antibody
in Cervical Mucus

Group 2

		undiluted	1:4
699A	30 min	-	D
	60 min	-	-
699B	30 min	D	-
	60 min	-	-
700A	30 min	-	D
	60 min	D	-
700B	30 min	D	D
	60 min	-	D
709A	30 min	D	D
	60 min	-	-
709B	30 min	-	-
	60 min	=	-
710A	30 min	-	-
	60 min	-	N
710B	30 min	-	-
	60 min	-	-
714	30 min	-	-
	60 min	-	-

Table 6

Sperm Microagglutinating Antibody
in Cervical Mucus

Group 3

		undiluted	1:4
701A	30 min	D	D
	60 min	D	D
701B	30 min	D	QNS
	60 min	D	QNS
704A	30 min	-	-
	60 min	D	-
704B	30 min	D	-
	60 min	D	-
706A	30 min	+	-
	60 min	D	-
706B	30 min	D	-
	60 min	D	N
712A	30 min	+1	+1
	60 min	+1	+2
712B	30 min	D	-
	60 min	D	N

Table 7

Sperm Macroagglutinating Antibodies in Serum

Group 1

	1:4	1:8	1:16	1:32	1:64
702A	2+	1+	1+	3+	3+
702B	3+	3+	3+	3+	3+
703A	1+	3+	3+	3+	3+
703B	3+	2+	2+	2+	2+
708A	3+	2+	3+	1+	1+
708B	1+	1+	3+	2+	2+
711A	1+	1+	1+	2+	1+
711B	1+	1+	1+	3+	3+
713A	1+	2+	3+	3+	2+
713B	1+	1+	1+	1+	2+

Table 8

Sperm Macroagglutinating Antibody in Serum

Group 2

	1:4	1:8	1:16	1:32	1:64
699A	+1	+1	+1	+1	+1
699B	+3	+3	+1	+1	+2
700A	+2	+3	+3	+1	+;
700B	+1	+2	+3	+1	+1
709A	+2	+1	+2	+1	+1
709B	+1	+1	+1	+1	+1
710A	+1	+1	+1	+1	+1
710B	QNS	+3	+3	QNS	+3
714A	+3	+3	+2	+2	+2
714B	+3	+2	+2	+2	+2

Table 9

Sperm Macroagglutinating Antibodies in Serum

	Group 3				
701A	+1	+1	+1	+1	+1
701B	+2	+2	+2	+2	+2
704A	+3	+1	+1	+1	+1
704B	+1	+3	+1	+2	+2
705	+1	+3	+2	+2	+2
706A	+1	+1	+1	+3	+1
706B	+1	+2	+1	+1	+1
712A	+1	+1	+1	+1	+2
712B	+1	+1	+2	+1	+1

Table 10

Sperm Immobilizing Antibodies
in Cervical Mucus

Group 1

		numbers of sperm alive	numbers of sperm dead	S.I.V.
702A	without complement	82	18	
	with complement	3	97	.18
702B	without complement	52	48	
	with complement	12	88	.55
711	without complement	54	46	
	with complement	13	87	.53

Table 11

Sperm Immobilizing Antibodies
in Cervical Mucus

Group 2

		number of sperm alive	number of sperm dead	S.I.V.
710A	without complement	60	40	
	with complement	10	90	.44
710B	without complement	23	77	
	with complement	7	93	.82
714	without complement	85	15	
	with complement	1	99	.15

Table 12

Sperm Immobilizing Antibodies
in Cervical Mucus

Group 3

		number of sperm alive	number of sperm dead	S.I.V.
701A	without complement	80	20	
	with complement	6	14	.21
701B	without complement	57	43	
	with complement	69	31	1.4
706A	without complement	8	92	
	with complement	1	99	.83
706B	without complement	3	97	
	with complement	5	95	1.02

DISCUSSION

There are many reports in the literature dealing with the induction of sperm antibodies in female animals. In all cases the method used to induce sperm antibodies involved multiple injections (a minimum of two times weekly for 6 weeks) and higher numbers of spermatozoa (100,000,000) per injection that would be expected to be encountered in the induction of antibodies in the natural state. Several authors (1,45) combined parenteral injections along with infusion of spermatozoa into the vagina. Omran (51) inserted tampons containing spermatozoa into the cervix of cattle for 5 to 6 weeks and induced sperm antibodies in the vaginal secretions.

Some infertile women with no physical abnormalities have sperm antibodies present in serum and/or cervicovaginal secretions and it is postulated that these antibodies are related to the infertility (9,10,22,64).

There are no references to immunologic infertility of the bitch in the literature. Sperm appears to have the potential to produce antibodies in the bitch which may cause infertility or decreased litter size. The purpose of this project was to produce sperm antibodies in the bitch using an innoculation procedure and a volume of semen which would be similar to natural conditions. Another purpose was to determine if the addition of bacteria to the semen would increase the incidence of sperm antibodies. Such an innoculation could occur if there was a vaginal laceration during coitus. The presence of a vaginal infection would provide the bacteria, or they would derive from the normal bacterial population as is present at all times. There is evidence that a vaginal infection could predispose women to the formation of sperm antibodies (11). This may hold true in the bitch.

Group one (control) bitch 702 was the only bitch to have a sample positive greater than the 1:8 dilution. This may be a result of the increased amount of hormones present since the sample was taken on day 10 of the estrous cycle. It has been shown that a positive sperm agglutination test can occur with increased levels of the beta lipoprotein which carries sex hormones (60).

Group two (semen) had two bitches with a positive agglutination at 1:16. These bitches also showed a larger agglutination per sample. Bitch 714 had a large number of agglutinated sperm in the preinjection sample. The agglutination appeared to be a nonspecific reaction.

Bitch 701 was the only animal in group three who increased the agglutination titer and degree of agglutination observed in the preinjection as compared to the post-injection samples. All other bitches had negative results.

The sperm microagglutination test of serum did not produce consistent positive results in group two or three. There were many insignificant positive results especially in the control group. Ogra & Ogra (50) showed that intravaginal injections produce only transitory serum antibodies and this may account for the negative results. The low numbers of spermatozoa injected (40,000) and the small injection schedule (two times) may have failed to provide an adequate stimulus for the production of systemic antibodies to sperm.

The sperm microagglutination test of cervical mucus was difficult to interpret because most samples contained cellular debri from the vagina. This tended to cause frequent nonspecific agglutination. The control group (one) showed a greater degree of agglutination than did group two or three. It appeared that another agent besides sperm antibodies can cause agglutination

in cervical mucus. All samples in group two were negative. 712 was the only bitch in group three with positive agglutination. This agglutination occurred at both titers. It is interesting to note that all bitches in group three except 712A had dead spermatozoa in the undiluted sample. While the number examined provided too small a population to draw concrete conclusions, this may be a significant finding. It is known that mixing vaginal mucus with sperm in some infertile bitches will cause immobilization and death of the sperm. This finding may be related to the cervical mucus sperm microagglutination test.

The macroagglutination test of serum in this study showed a large positive reaction in the control group. The semen (group two) and semen and bacteria (group three) injected bitches showed a predominantly negative reaction at the dilutions greater than 1:8. This test provided no useful information. It is not known why the positive reaction occurred predominantly in the control group or why very few positive reactions occurred in the semen and semen and bacteria injected bitches.

Good evaluation of the sperm immobilization test cannot be made since the complement used had a spermatotoxic reaction. This reaction caused from 88 - 99% immobilization and death of the spermatozoa in all of the groups. The complement which was added to the samples was fresh guinea pig serum. Hemolysis had occurred during the collection and the hemolysis of the red blood cells may have released a substance which was toxic to spermatozoa.

The poor staining of the vaginal smears made a proper evaluation of the results impossible. It is not known if the poor staining qualities were due to technician error or an inherent difficulty with the stain. The Sano stain has since proven to be difficult to properly reproduce by other workers in this college.

There was no difference in the organisms cultured between the three groups. All of the bitches had variation in the bacterial species present in the anestrous and the proestrus culture. However the bitches injected with the killed E. coli and streptococcus (group one and three) had no change in numbers of those organisms compared to the group injected with semen only (group two). This is not a conclusive finding however since the number of each organism present may be more significant than the presence alone. The change of bacterial species in the bitches may be influenced by two factors: the bitches were originally cultured upon arrival in a new environment and the vaginal flora may reflect on adaptation to the environment. The stage of the estrous cycle may also be a cause for the difference. The physiology and chemistry, especially the pH, change as the estrous cycle continues. The pH especially is a reflection in the changes in the hormonal status of the bitch. This change of pH would change the microfloral population.

CONCLUSION

There is some indication that spermatozoa has the potential to induce antibody in the bitch. The role of sperm antibodies in relation to infertility is undefined and needs further study. Induction of sterility by innoculation with semen also requires more work; however such a procedure would probably prove impracticle for broad implementation.

The sperm microagglutination test and the macroagglutination test of serum proved to be of no value in this study since considerable nonspecific agglutination occurred in the titers tested. The sperm microagglutination test of cervical mucus shows promise in providing a diagnostic aid for immunologically caused infertility. Additional work is needed in this area.

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APPENDIX

Table 13

Vaginal Cultures

PROESTRUS

ORGANISM	702	703	708	711	713	701	704	705	706	712	699	700	709	710	714
Pseudomonas diminuta				X											
P. putida	X														
P. maltophilia															
Pasteurella multocida	X	X	X	X	X		X		X	X		X	X	X	X
P. pneumotropica												X			
P. species				X											
Escherichia coli	X		X								X		X	X	
Proteus mirabilis											X				
P. morganii			X												
Aeromonas hydrophila	X														
Flavobacterium IIB										X					
Bacteroides melanogenicus		X					X		X			X			
B. species															
Neisseria subflava							X								
Acinetobacter calcoaceticus var. lwoffi										X					
Moraxella equi			X	X						X					
Staphlococcus aureus			X	X							X				X

Table 13 (Cont'd)

ORGANISM	702	703	708	711	713	701	704	705	706	712	699	700	709	710	714
S. epidermidis		X		X		X			X	X	X	X			X
Micrococcus species				X		X			X	X	X		X		
Group D Streptococcus		X		X		X			X	X	X	X	X		X
Clostridia perfringens															
C. sporogenes															
Bacillus subtilis															
B. cereus															
B. cersus Var. mycoides															
B. pumulis															
Lactobacillus acidophilus						X			X		X				X
L. leichmannii												X			
L. jensenii															
L. salivarius															
Streptomyces species															
Actinomyces viscosus											X				
Mycoplasma species															
IV-E						X	X								
II-J						X	X								

Table 13 (Cont'd)

ORGANISM	702	703	709	711	713	701	704	705	706	712	699	700	709	710	714
Streptococcus I		X								X					
Streptococcus II	X				X		X						X		
Streptococcus III		X		X			X							X	
Streptococcus IV	X													X	
Streptococcus V						X									
Streptococcus VI				X						X	X				
Corynebacteria I						X			X			X	X		
Corynebacteria II	X						X			X					X
Corynebacteria III		X	X									X			
Corynebacteria IV				X											
Corynebacteria V		X		X						X					
Corynebacteria VI															
Gram pos. anaerobe 1				X											
Gram pos. anaerobe 2				X											
Gram pos. anaerobe 3			X				X								
Gram pos. anaerobe 4			X												
Gram pos. anaerobe 5					X										
Gram pos. anaerobe 6															X

Table 13 (Cont'd)

ORGANISM	702	703	708	711	713	701	704	705	706	712	699	700	709	710	714
Gram pos. anaerobe 7															X
Gram pos. anaerobe 8														X	
Gram pos. anaerobe 9														X	
Gram pos. anaerobe 10														X	
Gram pos. anaerobe 11	X													X	
Gram pos. anaerobe 12										X					
Gram pos. anaerobe 13	X														
Gram pos. anaerobe 14		X													
Gram neg. anaerobe 1				X											
Gram neg. anaerobe 2			X												
Gram neg. anaerobe 3					X										
Gram neg. anaerobe 4					X										
Gram neg. anaerobe 5														X	
Gram neg. anaerobe 6														X	
Gram neg. anaerobe 7										X					
Gram neg. anaerobe 8		X													

Table 14

Vaginal Cultures

ANESTRUS

ORGANISM	702	703	708	711	713	701	704	705	706	712	699	700	709	710	714
Pseudomonas diminuta						X									
P. putida															
P. maltophilia		X													
Pasteurella multocida						X									
P. pneumotropica															
P. species					X										
Escherichia coli															
Proteus mirabilis			X												X
P. morganii															
Aeromonas hydrophila															
Flavobacterium IIB															
Bacteroides melanogenicus															
B. species					X		X								
Neisseria subflava															
Acinetobacter calcoaceticus var. lwoffi															
Moraxella equi															
Staphlococcus aureus	X	X	X	X	X	X	X	X	X	X				X	X

Table 14 (Cont'd)

ORGANISM	702	703	708	711	713	701	704	705	706	712	699	700	709	710	714
S. epidermidis	X	X	X	X	X	X	X	X		X	X	X	X		
Micrococcus species	X			X	X	X		X		X	X			X	X
Group D Streptococcus	X	X	X	X	X	X	X	X	X		X	X	X	X	X
Clostridia perfringens				X		X				X					
C. sporogenes	X														
Bacillus subtilis													X		
B. cereus	X														
B. cersus Var. mycoides	X														
B. pumulis	X														
Lactobacillus acidophilus	X	X				X	X			X	X			X	
L. leichmannii												X	X		X
L. jensenii													X		
L. salivarius														X	
Streptomyces species													X		
Actinomyces viscosus															
Mycoplasma species								X			X				
IV-E				X											
II-J						X									

Table 14 (Con't)

ORGANISM	702	703	709	711	713	701	704	705	706	712	699	700	709	710	714
Streptococcus I								X		X					X
Streptococcus II											X				
Streptococcus III		X		X		X		X	X	X	X				X
Streptococcus IV			X	X	X	X			X	X	X				X
Streptococcus V				X			X								
Streptococcus VI	X	X		X			X	X	X	X	X	X	X		
Corynebacteria I															
Corynebacteria II						X								X	
Corynebacteria III															
Corynebacteria IV									X	X					
Corynebacteria V				X											
Corynebacteria VI															
Gram pos. anaerobe 1															
Gram pos. anaerobe 2															
Gram pos. anaerobe 3															
Gram pos. anaerobe 4															
Gram pos. anaerobe 5															
Gram pos. anaerobe 6															

Table 14 (Cont'd)

ORGANISM	702	703	708	711	713	701	704	705	706	712	699	700	709	710	714
Gram pos. anaerobe 7															
Gram pos. anaerobe 8															
Gram pos. anaerobe 9															
Gram pos. anaerobe 10															
Gram pos. anaerobe 11															
Gram pos. anaerobe 12															
Gram pos. anaerobe 13															
Gram pos. anaerobe 14															
Gram neg. anaerobe 1															
Gram neg. anaerobe 2															
Gram neg. anaerobe 3															
Gram neg. anaerobe 4															
Gram neg. anaerobe 5															
Gram neg. anaerobe 6															
Gram neg. anaerobe 7															
Gram neg. anaerobe 8															

Table 15

Vaginal Smear

Group One (control)

	day of estrous	debri	noncornified cells	cornified cells	white blood cells	red blood cells
702	5	1+	28	72		
	7	1+	14	86		
	9	1+	5	95		
	10	1+		95	4	1
	12	1+	3	97		
703	5	1+	31	64		
	7	1+	22	31		47
	9	1+	9	71	17	3
	10	2+	36	63	1	
	12	2+	20	70	10	
708	5	1+	69	21	2	8
	7	3+	14	86		
	9	1+	16	84		
	10	1+	16	83	1	
	12	1+	17	82	1	
711	5	1+	12	87		1
	7	1+	11	89		
	8	1+	12	77		11
	9	1+	37	63		
	12	1+		99		1
713	5	2+	36	34	4	26
	7	3+	-	64	4	7
	9	1+	1	79	2	11
	10	1+	2	98		
	12	2+	9	71		5

Table 16

Vaginal Smears

Group Two (semen)

	day of estrous	debri	noncornified cells	cornified cells	white blood cells	red blood cells
700	6					
	7	1+	35	65		
	9	1+	36	61		3
	10					
	12	2+	4	25	43	28
709	7	1+	48	52		
	9	1+	26	74		
	12	1+	14	86		
710	5	1+	26		1	
	7	1+	16	66	2	16
	9	2+	14	85	1	
	10	1+	12	85	3	
	12	1+	8	86	3	3
714	5	1+	34	66		
	7	1+	34	66		
	9	1+	22	79		1
	10	1+	4	88		8
	12	1+	4	94		2

Table 17

Vaginal Smear

Group Three (semen plus bacteria)

	day of estrous	debri	noncornified cells	cornified cells	white blood cells	red blood cells
701	7	2+	8	56	16	20
	9	1+	19	76	2	4
	12	1+	5	86	1	8
704	5	1+	28	72		
	7	1+	72	22		
	9	1+	17	83		
	10	2+	32	65		3
		12	1+	30	70	
712	5	1+	3	77	1	1
	7	1+	16	80	2	1
	12	1+	1	91	7	1

Table 18

Known Positive Control

Microagglutination Test

	U	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
30 min.	D	D	D	D	D	3+	3+	2+	2+

Macroagglutination Test

1:4	1:8	1:16	1:32	1:65
3+	3+	3+	3+	3+

D all spermatozoa dead

INDUCTION AND DETECTION OF SPERM ANTIBODY IN THE BITCH

by

COLLEEN M. O'KEEFE

D.V.M., University of Illinois, 1974

An Abstract of a Master's Thesis

submitted in partial fulfillment

of the requirements for the degree

MASTER OF SCIENCE

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1977

ABSTRACT

Evidence of sperm antibodies in the bitch has never been reported. However, natural and induced sperm antibodies have been reported in women and various species of animals. The objective of this study was to induce sperm antibodies in the bitch using a method which would simulate natural exposure. Fifteen Beagle bitches were divided into three groups. One group had sperm injected intravaginally, one group received sperm and killed bacteria intravaginally and one group served as a control.

Three tests for sperm antibodies in the serum and the cervical mucus were used: the sperm microagglutination test of serum and cervical mucus, the macroagglutination test of serum, and the sperm immobilizing test of cervical mucus. The sperm microagglutination test and macroagglutination test of serum proved to be of no value in this study. The sperm microagglutination test of cervical mucus shows promise in providing a diagnostic aid for immunologically caused infertility. Additional work is needed in this area.