STUDIES ON
ANTI-SEMINAL ANTIBODIES AND THEIR
RELATION TO INFERTILITY IN LIVESTOCK

THESIS
Submitted To Magadh University In Partial
Fulfilment Of The Requirements For The
DEGREE OF
MASTER OF SCIENCE (VETERINARY)

By
S. P. Pandey, B.V.Sc. & A.H.
POST-GRADUATE DEPARTMENT OF GYNAECOLOGY & STERILITY
BIHAR VETERINARY COLLEGE, PATNA
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Dated, December, 1966.

I certify that this Thesis entitled
"STUDIES ON ANTI-SEMINAL ANTIBODIES AND THEIR
RELATION TO INFERTILITY IN LIVESTOCK" has been
prepared under my supervision by Shri S.P. Pandey,
a candidate for the M.Sc. (Vet.) with Gynaecology,
Obstetrics and Artificial Insemination as major
subject, and that it incorporates the results of
his independent study.

( B.P.S.R. Singh ).
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(S. P. Pandey)
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INTRODUCTION
INTRODUCTION

Infertility in domestic animals has long been recognised as the greatest bottleneck in the efficient livestock production, which has so vital a role in the economic welfare of a country, particularly so in relation to India having an agriculture-based economy, by producing working bullocks for the fields and meat, milk and other by-products for direct human consumption. Though the precise information about the magnitude of losses incurred by the livestock industry in India due to sterility are not available, it can well be imagined by an estimate of Asdell (1965) in terms of money for New York States and U.S.A. as 20 million and 250 million dollars respectively.

This, therefore, has engaged the attention of many observers and research workers in the field and a lot of opinions, observations and experimental results have been reported on the problem of infertility. But it still continues to be a baffling problem giving new avenues for research.

However, the factors held responsible so far for infertility are genetic, hormonal, nutritional and of microbial origin like Brucellosis, Vibriosis, Trichomoniasis and other bacterial and viral infections (Roberts 1956). There are also some other unestablished reasons due to which a cow or heifer although possessing normal oestrous cycles and without revealing any physical
and clinical abnormality on examination, fails to conceive after being served by normally fertile bulls. Such cases of repeat breeding have attracted attention of the research workers (Tanabe and Casida, 1949; Kidder et al 1954) and have been characterised by failure of fertilization or early embryonic death. Although it seems that much work has been done to study the factors leading to fertilization failure or early embryonic death, still a definite and concrete explanation has not yet been put in the light of previous studies.

The report of Landsteiner (1899) and Metchnikoff (1899) that the mammalian semen and its constituents, chiefly the sperms were antigenic, opened a new field for the research workers to throw light on antigen-antibody reactions in relation to infertility and sterility. The subsequent available informations (Docton et al, 1952; Brabanov & Dikov, 1959; Menge et al, 1982) could lead to a presumption that in cows the antibodies might be produced against sperm or seminal components and these in turn might react with the respective antigens in subsequent inseminations, as a result of which such antigen-antibody reactions might be responsible for the failure of fertilization or early embryonic death.

The investigations on immunological factors associated with sterility in farm animals have been receiving increasing attention during recent years. However, works so far carried out on this aspect are very scanty and that too mostly in laboratory animals.
Therefore, the present investigation was undertaken with a view to study the antigenicity of buck semen and its possible role in immunological infertility. Various antigen-antibody systems were tried to find out a suitable one to diagnose the cases of immunological infertility in cattle. It is hoped that this investigation will, in some measures, lead to valuable information in the diagnosis of immunological infertility in repeat breeding cows and further this method may be applied to locate a suitable bull for successful insemination of such repeat breeding cows.
REVIEW OF LITERATURE
REVIEW OF LITERATURE

Antigenic nature of semen and its constituents:

Landsteiner (1899) reported for the first time that spermatozoa were antigenic. He observed that bull spermatozoa rapidly became immobile when injected into the peritoneal cavity of guinea pigs that had previously been injected parenterally with bull spermatozoa. This was further confirmed by Metchnikoff (1899) who tested for agglutinins and sperm immobilizing antibodies.

Metchnikoff (1900) proved that guinea pigs could produce antibodies against spermatozoa of the same species of animals. Von Moxter (1900) injected ram sperm into rabbits and obtained antisera which were spermicidal to rat sperm.

Farrum (1901) found that rabbit injected intra-peritoneally with semen or testicular emulsions of dog, bull or man developed specific antibodies for each kind of material. Pfeiffer (1901) injected rabbits with dried and powdered bull sperm extracts. The resulting antiserum reacted strongly with semen solutions and testis extracts and negligibly if at all with extracts of other bovine organs.

Struve (1902) obtained precipitins by injecting rabbit with human semen and testicular extracts.

Nekton and Manly (1923) injected human seminal fluid and sperm as well as swine, bovine and equine seminal fluids into rabbits and reported species and semen-specific precipitating indicating that the
seminal fluids were antigenic.

McCartney (1923) injected female rats subcutaneously with spermatozoa suspension and detected by sperm agglutination test the circulating antibodies as well as antibodies present in vaginal and uterine secretions. Landsteiner and Levine (1926) demonstrated that the sperm cells of human being of the appropriate blood type would absorb specifically and almost completely the immune antibodies (from rabbits) to the A and B antigens of human erythrocytes.

Pomeranke (1928) demonstrated that serum as well as the vaginal secretions of female rabbits injected with rabbit sperm or testicular extract were toxic for rabbit sperm. He observed cross-reactions between rabbit and rat sperm.

Mudd and Mudd (1929) demonstrated that mammalian spermatozoa possessed both species and tissue specificity. He proved that antibodies prepared in rabbits against spermatozoa of bull, ram, guinea pig, rat and man were species specific. Cross reactions occurred between bull and ram spermatozoa and their corresponding antisera as well as between anti-ram sperm sera and guinea pig spermatozoa.

Henle (1938) supported the contention that species - specificity of spermatozoa was of dominant rather than absolute nature, for he found cross-reactions between sperm of different species; there was a strong
antigenic resemblance between bull and sheep and less reactivity between bull and man. Also all rabbit anti-bull sera exhibited marked reactions with human sperm.

Henle et al (1938) conducted extensive studies on the antigenic nature of mammalian spermatozoa and characterized especially the antigens of bull sperm. They found (i) heat-labile, head specific and tail specific antigens and a heat-stable antigen common to both heads and tails that was species specific, (ii) three different cross-reacting antigens, two of which were in the heads and one in the tails and (iii) one head antigen that was not active in the native cells (discovered after rupture of the sperm).

Parsons and Hyde (1940) produced anti spermatozoal antibodies in rabbits, against spermatozoa of ox, sheep, guinea pig, rat and rabbit. They demonstrated the antibodies by different serological tests proving that spermatozoa of the respective species were antigenic.

In an attempt to characterise the proteins of human seminal plasma, serologically, Ross (1946) prepared antibodies against human seminal plasma in rabbits.

Chang (1947) found an unstable thermolabile spermicidal factor present in fresh human, bovine, rabbit, guinea pig, and rat sera. It killed the spermatozoa of its own species and the sperms of other species. This factor was not present in tissue extracts and various plasma protein fractions. This factor which has several
characteristics similar to those of complement, varied in strength or concentration in different individuals and in different species.

Smith (1949 a) provided evidence of the antigenic properties of mammalian spermatozoa and demonstrated different types of agglutination by antisperm antisera. Smith (1949 b) further produced antiserum in a goat against rabbit seminal plasma which reacted with seminal plasma, but did not agglutinate rabbit spermatozoa, whereas antispermatozoal sera did cross react with seminal plasma.

Docton et al (1952) demonstrated the antigenicity of bovine spermatozoa by immunizing sheep with the washed spermatozoa of bull. He found that iso-immune serum, containing antibodies for bovine erythrocytes also reacted specifically with bovine spermatozoa. Antibodies produced in sheep against bovine spermatozoa caused agglutination of bovine spermatozoa and also produced specific lysis of erythrocytes of certain cattle.

Larson et al (1954) conducted ultracentrifugal, and immunological studies of bovine seminal plasma proteins and indicated that they were highly antigenic. Chemical, electrophoretic, ultracentrifugal and immunological comparisons revealed that the major proteins of seminal plasma were distinct entities which were either absent from or were only minor constituents of blood or milk serum.
Weil et al. (1956) for the first time studied the immunological properties of human seminal plasma and the peculiar relations of the antigens of seminal plasma to those of human spermatozoa. He showed that seminal plasma contained highly antigenic material. He could not distinguish between seminal plasma and spermatozoa by the immunological technics employed. He speculated that antigenic materials originated from the fluid products of the genital tract rather than from the spermatozoa, because ejaculates free of spermatozoa showed the same immunological behavior as does seminal plasma from normal semen.

Pernot (1956), using immuno-electrophoresis found that guinea pigs seminal plasma contained 11 antigenic constituents while guinea pig sperm tail extracts had seven constituents. Some of the antigenic constituents were common to blood serum, seminal plasma and spermatozoa.

Gulbring (1957) studied the antigenicity of human spermatozoa and concluded that spermatozoa contain substances identical with or similar to the blood factors of erythrocytes.

Whether or not spermatozoa were themselves antigenic was questionable in light of the work of Weil and Finkler (1958) who found that both guinea pig anti-rabbit seminal plasma and anti-rabbit sperm immune sera strongly agglutinated spermatozoa from fresh semen as well as thrice washed rabbit spermatozoa. The antigens of rabbit spermatozoa and seminal plasma were so closely
related that they could not be differentiated.

Katsh (1959) speculated that hyaluronidase might be one of the antigens present in the spermatozoa. In view of the lack of cross-reactivity between bull testicular hyaluronidase and guinea pig testicular hyaluronidase and because neither of these enzymes cross-reacted with staphylococcal hyaluronidase, it was clear that these enzymes were distinctly different antigenic entities.

Rao and Sadri (1959) working with human semen demonstrated fifteen and sixteen antigens in human semen and seminal plasma. They also reported that seminal plasma and cervical mucus had antigens common to blood serum.

Baum et al (1959) found that guinea pigs of both sex if injected with sperm or testis with Freund's adjuvant, developed antibodies which immobilized sperm in the presence of complement. Baum (1959) demonstrating the reaction of guinea pig spermatozoa with homologous antibody by fluorescent antibody staining found that such antibodies reacted with the heads of mature guinea pig spermatozoa but not with the precursor germ cells.

Weil and Finkler (1959) studied the iso-antigenicity of rabbit semen. They found that rabbit seminal plasma contained at least one iso-antigenically active component. No iso-antigenicity could be observed with rabbit spermatozoa under comparable experimental conditions.
Edwards (1960) showed that rabbits absorbed enough antigen from semen introduced into the vagina to give an antibody response, although positive results were obtained only with heterologous (bull) semen, but not with homologous ones. He reported that sperm agglutinins and sperm immobilizing antibodies as well as precipitin could be detected in the serum but not in the genital tract of rabbits receiving intra muscular injection of semen and adjuvant.

Weil and Rodenburg (1960) stated that human spermatozoa from spermatoceles were lacking in antigenic material present on seminal spermatozoa, which these latter cells share with the seminal plasma, thereby proving that human spermatozoa acquire antigenic material during their passage through the adnexal glands of the male genital tract.

Weil (1960) showed that rabbit spermatozoa from the epididymis lacked the antigenic material present in the seminal spermatozoa, which these latter cells had in common with the seminal plasma. This observation provided further support for the indirect evidence, obtained previously, that antigenic material is also taken up by the spermatozoa from the seminal plasma.

Rao and Sadri (1960), using agar-gel-diffusion test, found that buffalo seminal plasma contained 16 antigens and that epididymal and ejaculated spermatozoa had atleast 7 antigens, three of which were not common to
either seminal plasma or blood serum.

Weil (1961) found that human seminal plasma contained strongly antigenic material that was species and organ specific and which originated from seminal vesicles. During the passage of spermatozoa through the male genital tract, these antigens become firmly attached to them.

Gordon and Hunter (1961) while studying the spermatozoa-specific antigens in the bovine, employed complement fixation and agar-gel-diffusion test. They stated that bull spermatozoa contained at least seven antigens out of which only one was specific for spermatozoa.

Weil and Rodenburg (1962) studied the antigenicity of human and rabbit seminal plasma by fluorescent antibody technique. They found that only the seminal spermatozoa took the fluorescein-conjugated antise seminal plasma immune globulin stains. Testicular spermatozoa could not be stained with the reagent for spermatozoa coating antigen. Their study of the different parts of male genital tract revealed that spermatozoa coating antigen is produced in the seminal vesicle.

Beck et al (1962) employing immuno fluorescence antibody technique to study reactions between serum and spermatozoa from guinea pigs, rabbits, mice and men demonstrated two distinct reactions of immunological nature between serum and spermatozoa. The reaction
between serum and the acrosome occurred with serum from normal animals, whereas that between serum and sperm tail occurred only after the immunization of guinea pigs with homologous spermatozoa.

Shahani and Southam (1962) working on antigenicity of human spermatozoa, showed the presence of blood group antigens A or B on human spermatozoa with the use of specific immunofluorescent antisera.

Bratanov and Dikov (1963) found that seminal fluid had a higher antigenic activity than spermatozoa. They did not find any antibody against bull and ram spermatozoa in normal rabbit sera, but after active immunisation with bull spermatozoa, such antibodies were produced. There was no antigenic similarity between the spermatozoa and erythrocytes of bull, ram and stallion.

Hathaway and Hartree (1963) observed 4 precipitin lines between extracts of ram acrosomes and an antiserum to those extracts. However, the 4 lines appeared to be continuous with some of the six lines that formed with ram seminal plasma and none of them appeared after absorption of the antiserum with seminal plasma.

Edwards et al (1964), using the mixed agglutination and mixed antiglobulin reactions showed that the spermatozoal membrane possessed species specific antigens in common with red blood cells.

The antigenicity and cross reactions of bovine seminal constituents were studied by Hunter and Hafs (1964). They found that bovine ejaculated spermatozoa, seminal plasma and blood-circulating antigens contain antigens.
semenal plasma and blood serum contained common antigens. Ejaculated spermatozoa possessed at least seven antigens. Five of these were shared with seminal plasma and at least one with blood serum. Absorption of anti-sperm immune sera with seminal plasma and blood serum revealed that epididymal and ejaculated spermatozoa shared at least three protein antigens which were not found in seminal plasma or blood serum.

Matousek (1964a) repeated the investigations of Docton et al. (1952) but was unable to demonstrate the presence of the erythrocytic antigens of cattle either in the seminal plasma or in the epididymal spermatozoa of bulls of red spotted breed. The negative results were not altered by mechanical fragmentation or by enzymic disruption of the spermatozoa.

Matousek (1964b) further studied the antigenic characteristics of bull spermatozoa as revealed by the action of agglutinins present in normal and immune sera. In the normal sera of bulls, rams and sows, antibodies agglutinated not only homologous but also heterologous spermatozoa. The absorption of normal sera with homologous spermatozoa suppressed the reaction against homologous and heterologous germ cells. However, heterologous spermatozoa could absorb agglutinins only against spermatozoa of their own species. Antibodies obtained by immunizing rabbits with bull and ram spermatozoa not only agglutinated homologous spermatozoa, but also gave cross-
reactions with germ cells of other species. Similarly antibodies against bull seminal plasma agglutinated not only bull but also ram spermatozoa.

Matousek (1964 c) in other studies demonstrated the antigenicity of bull spermatozoa by some biochemical and immuno-electrophoretic methods in which he supported that both bull spermatozoa and seminal plasma have specific antigens and common or cross-reacting antigens. On agar plates, antiseminal plasma antibodies formed five arcs with the seminal plasma of bulls. The fructolysis index of bull seminal spermatozoa was reduced in the medium of the antisera containing antibodies against seminal and epididymal spermatozoa.

Rao and Bangalore (1964) conducted immunological investigations with rabbit semen by agar-gel-diffusion tests. They found that rabbit seminal plasma, spermatozoa and gel mass had six, four and four antigens respectively.

Agar et al (1965) using agar-gel-diffusion test demonstrated that ram semen contained 12 antigens and ram seminal plasma had 10 antigens out of which two antigens were common to both. With paper electrophoretic studies, a significant rise in gamma globulin fraction of antisera was noted.

Bennette (1965) found striking species specificity between seminal plasma proteins of bull, ram, rabbit and Boar by agar-gel-electrophoresis.
Cavaller (1965) studied the antigenic properties of bovine and ovine semen, spermatozoa and seminal fluid by injecting them intravenously into rabbits. He found that in either species whole semen was more antigenic than spermatozoa, which in turn were more so than seminal fluid. The antibodies agglutinated the homologous spermatozoa. In either species, spermatozoa and seminal fluid had common antigens.

Mittal et al (1965) studied the antigens of bovine semen and noted the influence of specific rabbit anti-bull-semen serum on metabolic activity of spermatozoa. They detected at least 20 antigenic components in bull semen by micro-immuno-electrophoresis and also noted drastic reduction in oxygen uptake, fructose utilization and lactic acid production by bull spermatozoa when mixed with specific antiserum.

Stevens et al (1965) using the haemagglutination technique found antibodies to human seminal plasma in 30% of the sera from hospitalized patients. They also produced antibodies against human seminal plasma in rabbits which reached in haemagglutination tests.

Seminal antibodies and infertility:

A. Female infertility:— Literatures on the female infertility can be grouped under two headings (i) due to seminal antibodies (ii) due to erythrocytic antibodies.
(1) Due to seminal antibodies.

The scientific beginning of this topic dates back to 1899-1900 when Landsteiner (1899) and Metchnikoff (1899) produced for the first time, antibodies against sperm or testicular extracts of bull by injecting it into guinea pig.

Savini (1911) attempted to induce sterility in female rabbits and guinea pigs with sperm injections. He exposed the immunized females for 10-15 days to males one week after the last injection. No youngs were obtained from this exposure, but many fertile matings resulted from subsequent copulations. Venema (1916) cited experiments in which sterility resulted in female rabbits that were injected with testis.

By injecting the semen of rabbits or of closely related species, Dittler (1920) rendered female rabbits sterile for varying period depending upon the number of injections given. In order to eliminate the possibility of a primary sterility due to other causes, he used only those rabbits which had given birth to at least one litter. He found the blood serum of the sterilized animals to be definitely toxic to the spermatozoa, causing agglutination and inhibition of their motility.

Guyer (1922) produced sterility in rabbits and guinea pigs by injecting them intravenously with the spermatozoa. Also the serum of the injected animals became spermatoxic.
Vogt (1922) demonstrated antibody formation against human spermatozoa following absorption of semen through the genital tract. He stated on clinical grounds that overloading with seminal products may lead to sterility and that natural fecundity restored by abstinence from sexual intercourse. The first cohabitation was the one that most frequently resulted into pregnancy. Mayer (1922) was of the opinion that abortion of very young embryo may result from frequent coitus after a long period of abstinence in human being.

McCarty (1923) found that the female rats injected subcutaneously with spermatozoa suspension remained sterile for a period of two to 22 weeks beyond the normal gestation time, although the normal sexual cycle and sex behavior of the animal was not affected. He found spermatoxins in vaginal and uterine secretions of the immunized animals which had agglutinating effect upon the spermatozoa.

Castoro (1926) induced temporary sterility for a period in guinea pigs by subcutaneous injections of the seminal products of male guinea pigs. The injection were well tolerated and no anaphylactic reactions were observed in any instances.

Fogelson (1926) described an accurate method of inhibiting the conceptions temporarily in rats by sensitizing the female to spermatozoa protein. Jarcho (1926) found that female rabbits injected with spermatozoa
suspension from ram and guinea pig were sterile even after seven months. The serum from immunized rabbits contained spermatoxin but he was unable to find evidence of spermatoxic substances in the vaginal secretion.

Kostromin and Kartashev (1927) performed the most exhaustive experiments with reference to biologic induction of temporary sterility in female animals. They injected female rabbits and guineapigs with heterologous spermatozoa, both living and dead and obtained positive result in both cases. However the results were better when living spermatozoa were used.

Pomeranke (1928) found that intravaginal injection of rabbit sperm into female rabbits led to detectable antibodies in the serum as well as in the vaginal secretions of the injected rabbits, but he was unable to obtain conclusive evidence that sterility could be induced by intravaginal injection of sperm.

Ardelt (1931) in his experiments with female rabbits reported that injection of sperm resulted in temporary sterility. Baskin and Enver (1932) proved the possibility of immunization of women with spermatozoa. The immunity lasted for one year and revaccination at the end of the year prolonged the immunity for at least another year. They observed that period and degree of immunity could be determined by the presence of antibodies in the blood.

Guyer and Clause (1933) produced temporary or permanent sterility in female rats by injecting a
nucleoprotein fraction prepared from bull testis. Wang (1936) injected sperms of rats, dogs and sheeps into female rats and although he was unable to confirm previous reports of induced sterility, he did find the sera of the injected animals to immobilize and agglutinate sperm.

Henle and Hene (1940) immunized female guinea pigs with spermatozoa of bull. They got antibody response in 60 to 70% of the animals immunized with homologous spermatozoa and in 100% of the animals immunized with heterologous spermatozoa. Although the serum contained antibody against sperm there was no decrease in fertility nor a delay in conception in the immunized animals.

Henle et al (1940) conducted experiments with a view to determine whether or not temporary sterility in female white mice can be produced by passive immunization with antisera against spermatozoa of the mouse and the rat. Their results indicated the absence of any effect upon fertility.

Parsons and Hyde (1940) undertook an extensive study to evaluate the use of spermatoxic sera in the prevention of pregnancy. They demonstrated antibodies against ox, sheep, guinea pig, rat and rabbit spermatozoa by different serological techniques. They did not find any evidence that pregnancy could be delayed or prevented by treatment of rat or rabbit with sperm antigens. They demonstrated antibodies to guinea pig sperm in the blood of rabbits by intravaginal injection. They also found that
intravaginal injection of immune guinea pig serum temporarily arrested fertility of rabbits.

Brunner (1941) immunized female rabbits with a "bull sperm phospholipid" suspended in sheep serum and found that, although fertility was unimpaired, a small but significant percentage of offspring were hermaphrodite.

Garcia Alfonso and Perez Y Perez (1959) observed that female rabbits remained sterile till the concentration of sperm agglutinin in blood serum was over 1:100. Further they were able to produce similar mucous agglutinins and eventually serum agglutinins by daily insemination of female rabbits for 40 days. Similar sperm-agglutinins in infertile mares had been demonstrated by them and they considered 10% of infertility in mares was of this type.

Kidty et al (1959 b) in their study on the effects of semen treated with homologous antibodies on fertility in rabbits had shown that such semen on insemination could prevent fertilization and caused embryonic death. They observed further that the treatment of semen with dilutions of immune serum that permitted a near normal fertilization rate resulted in increased embryonic mortality in rabbits. One possible explanation was that the spermatozoa carried antibodies into the ovum at fertilization and these antibodies in some unknown way caused the death of the developing embryo.
Isozima et al. (1959) introduced Freund's adjuvant in the immunization of female guinea pigs with testis homogenate. He conclusively demonstrated the development of sterility in these immunized animals.

Kiddy et al. (1959 a) were the first who tried antigen-antibody reactions to demonstrate immunological causes of lowered fertility in cattle. Antibody could not be detected in the sera of 17 heifers after repeated intravenous injections of bull semen. Heifers exhibited anaphylactic reactions. There was no decrease in fertility of those heifers on breeding. They further tried to test the hypothesis for local antibody production as a factor in lowered fertility, for which they gave repeated intra-uterine injections of a bull's blood in 12 heifers. Six of the heifers showed antibody titer in their sera probably in response to the antigens which passed into the circulation, but the antibodies were not demonstrable in the reproductive organs through out the treatment period. On breeding no reduction in their fertility was found.

Bratnov and Dikov (1959) observed high titre of sperm agglutinins against bull semen in the sera of cows that were resistant to pregnancy. They indicated a direct correlation between the amount of these agglutinins in the cow serum and the conception rate.

Nakabayashi et al. (1961) examined blood serum of infertile human subjects for agglutinin against sperm. There was variation in agglutination titre with different
sperm specimens. Although the results varied with the sperm specimens used, a definite small percentage of infertile individuals possessed sperm agglutinating substance. Blood specimens of unmarried couples and of those married couples whose bloods were obtained just before or soon after conception were negative.

Bratanov and Dikov (1961) carried out washed spermatozoa agglutination test with oestral mucus, saliva, milk and cow blood serum, the latter giving the best reactions. Titres did not exceed 1:16 when conception occurred to first service (42 cows) but when cows (total of 48) returned repeatedly to a given bull, titres up to 1:512 were often obtained. Other bulls tested against this serum gave low titres and on service with these bulls conception resulted.

Studies conducted at the Agricultural Research Station and Agricultural Experiment Station of Wisconsin showed that cows can be made to produce antibodies against spermatozoa. When serum of cow which had previously been injected with bull semen, was added to bull sperm and used for insemination of six heifers, five came in heat about three weeks later but were infertile and one conceived but its embryo was deteriorating after 42 days of pregnancy (Report of U.S. Department of Agriculture, 1962).

Menge et al. (1962 a) produced immune sera in cows against bull semen and against bull sperm in rabbits. When bull semen was treated with these sera, prior to
insemination, it resulted into fertilization failure or possibly early embryonic death. Similarly fertilization was prevented in rabbits inseminated with semen treated with cattle anti-rabbit semen serum. Fertility occurred in both species when inseminated with semen treated with normal serum. They were able to prevent fertilization in rabbits inseminated with semen treated with gamma-globulin fraction of the immune serum, but not with gamma-globulin fraction of the normal serum. Further they indicated the absence of the cross-reactivity between the antigens of either seminal plasma or spermatozoa with erythrocytes. Antisera produced against erythrocytes had not antifertility effect.

Menge et al. (1962 b) studied the effects of cattle immune sera produced against rabbit semen and rabbit erythrocytes, on rabbit embryos of one and nine days of age in situ. They found that immune sera produced increased death rate among those embryos that were treated at nine days of age. Immune sera produced against erythrocytes and normal sera did not produce significant effect upon survival rates of embryos treated at either stage of development.

Gupta et al. (1962) examined the chemical and physiological properties of the cervical fluids from intact cows during oestrous. They found that fluids from repeat breeding cows tended to have lower spermatozoal preservation capacity than did the fluids from normal cows.
Behrman et al. (1963) studied in guinea pigs the effect of active and passive immunization with semen upon fertility. There was no reduction in fertility when non immunized female guinea pigs were artificially inseminated with washed epididymal spermatozoa pretreated with immune serum. They showed reduction in fertility rate in immunized female guinea pigs, when artificially inseminated. On mating of animals immunized against semen or insemination with sperm diluted in Lock's solution, the conception rate was 34.6%, as compared with 78.6% among control animals. The embryo count was concomitantly reduced from 23.6% to 7.9%.

Behrman & Otani (1963 a) immunized female guinea pigs with guinea pig testis and epididymal sperm by intravaginal route. They got positive uterine responses to the Dale-Schultze test in all animals immunised transvaginally. They suggested the presence of a tissue fixed antibody. In further studies Behrman and Otani (1963 b) postulated on the basis of the Dale-Schultze, precipitation and agglutination tests that homologous sperm agglutinins passed from the serum into the uterine and vaginal fluids and tended to fix in the uterus.

Wentworth and Mellen (1964) showed that spermatozoal antibodies developed in the domestic hens following repeated natural mating or artificial insemination and that natural immunity to spermatozoa had a detrimental effect on fertility.
McLaren (1964) conducted experiments related to immunological control of fertility in female mice. He found that degree of fertility in immunized female mice against spermatozoa was adversely affected. Degree to which litter size was depressed depended on the titre of agglutinating antibody in the serum. The lowered fertility of immunised female appeared to result from a reduction in fertilization rate due to failure of a proportion of spermatozoa to reach the oviduct.

Ashitaka et al (1964 b) found a high titre of sperm-immobilizing antibody in the vaginal secretion of guinea pigs immunized with homologous testis and sperm, but the titre was extremely variable as compared with that in the serum.

Isozima and Ashitaka (1964) could not find antibodies in non-immunized female guinea pigs after instillation of homologous sperm into their vagina or after exposure to male. He observed that antibody titre in female guinea pigs, which were well immunized with guinea pig testis was remarkably increased by the sperm instillation into the vagina or by exposure to a male.

Franklin and Dukes (1964) indicated by their studies a strong correlation between the existence of circulating antispermatozoal antibody and unexplained infertility in human being. They examined blood sera of 89 patients, whose fertility status had been determined, for the presence of circulating antispermatozoal antibody.
Antispermatozoal antibody was found in 78.9 percent of the females with no demonstrable organic causes for infertility, in 10.37 percent of the those with organic reasons for infertility, in 4.8 percent of the patients of known fertility and in 4.27 percent of the patients of unknown fertility.

Edwards (1964) made a study of fertilization in female mice immunized with homologous spermatozoa by subcutaneous or intraperitoneal injections of spermatozoa alone or combined with complete Freund's adjuvant. They did not find any decrease in mating performances by immunization but there was reduction in the fertility in the immunized group.

Ayalon (1964) also in his report in the 5th International Congress on Animal Reproduction, mentioned the role of immunological factors responsible for lowered fertility in cattle. He described these factors in connection with failure of fertilization and embryonic mortality in rodents, sows and ewes too.

Weil and Roberts (1965) immunized female rabbits with homologous seminal plasma in Freund's adjuvant. About 70% of the immunized animals had circulating antibodies against seminal plasma but this was not conducive to impairment of fertility.

Behrman & Nakayama (1965) studied the variation of antibody titre over an 8 month period in 5 rabbits following intradermal or intravaginal immunization with
homologous testis homogenate. They observed that during the period of high antibody titre, the female rabbits remained infertile even after several matings. Mating immediately after the disappearance of the antibody also failed to obtain a pregnancy. After a month of disappearance of antibody there was conception in female rabbits.

Bratanov et al (1965) concluded from tests on 79 cows that the increase in the sperm antibody titre of blood serum before the completion of uterine involution inhibited conception; the titre also increased in cows with latent endometritis. Insemination was not recommended when the sperm antibody titre exceeds 1:512.

Peterson (1965) observed that uterine secretions from repeat breeding cows had depressing effect upon motility and oxygen consumption of bull spermatozoa as compared to (fertile cows) easy breeders.

McLaren (1966) showed that the effect of sperm immunization on reduced fertility was more in randomly bred than inbred strains of mice. He noted titre of sperm agglutinins and reduction in litter size in randomly bred mice.

Katsh (1957 and 1958) used the Schultz-Dale technique and found that a guinea pig uterus immunized with testis or sperm would contract upon contact with a suspension of sperm. On the basis of this finding, he suggested that the contraction induced would impair the implantation of the fertilized egg in the endometrium.
Ashitaka et al. (1964 a) tested the suggestions made by Katsh (1957 and 1958) that uterine anaphylaxis might be a cause of sterility in guinea pigs immunized with testis and sperm, but they found this suggestion unsatisfactory from the standpoint of immunological desensitization as well as on the basis of the results of their own study.

(ii) Due to erythrocytic antibodies:

Since the antigenic relationship has been observed between the erythrocytes and spermatozoa (Landsteiner and Levine, 1926; Docton et al., 1952; Pernot, 1956; Gulbring, 1957; Helpern and Weiner, 1961; Shahani and Southam, 1962; Edward et al., 1964), antibodies against erythrocytes may also be a cause of infertility.

In species such as man and the horse, haemolytic disease of the newborn often occurred when maternal antibodies were present against the foreign erythrocytic antigen. This condition occurred in incompatible marriages. A possible etiological factor for unexplained infertility in anatomically and physiologically healthy couples could be the incompatibility of the ABO(H) blood system, where the antigens carried by the spermatozoa were blocked or immobilized by the antibodies present in the cervical secretions (Matsunaga, 1955; Gershowitz et al., 1958; Behrman et al., 1960).

The bovine placenta chorion is covered with multiple layers of epithelium. This prevents the passage
of bacterial antibodies but not of erythrocytic antibodies. Though haemolytic disease of cattle is unknown there are many unexplained instances of early abortion, macerated foetus, foetal hydrops and premature stillbirth.

Laing and Blackmore (1951) examined sera of 4 herds of cows of varying fertility against saline suspension of R.B.C. of bulls to which they were or had been in calf. They produced high titre of antibodies in the cows by intravenous injection of bull erythrocytes. Antibodies were also found in the colostrum of the immunized cows. They claimed that maternal circulating R.B.C. antibodies could cause abortion in cattle. Contrary to this Kiddy et al (1958) did not find any incidence of such incompatibility affecting fertility. He immunized the heifers by intravenous injections of the blood of a bull and the heifers were bred to that bull. These heifers had circulating antibodies for antigens of their calf's erythrocytes. Kiddy et al (1959 a) found circulating antibodies in six out of 12 heifers immunized against bulls erythrocytes. In contrast, there was no evidence of the presence of local antibodies in the tissues of the reproductive organs. When the 12 heifers were bred to the bull used as a blood donor, there was no indication of lowered fertility.

B. Male infertility:

Since Landsteiner (1899) and Metchnikoff (1899) independently discovered that it was possible to
induce the formation of antibodies against spermatozoa in guinea pigs by means of parenteral injection of heterologous sperm, several workers have explained male infertility to be caused by the presence of seminal antibodies.

Adler (1908) and Goyer (1922) demonstrated that guinea pigs or rabbits can produce antibodies even against their own spermatozoa, when injected with homologous sperm or testis suspension. Freund et al (1953) reported the formation of antibodies against testicular tissue in male guinea pigs. They studied the damage caused by the immunization in the testicular tissue of the male guinea pigs. The damage started with the inhibition of the maturation of spermatozoa leading through degeneration and exfoliation of spermatids, spermatocytes and finally to the spermatogonia and thus resulting into aspermatogenesis. Other part of the testicle remained unaffected. Wilson (1954) reported the presence of sperm agglutinins in the seminal plasma and blood serum of two sterile men up to titre of 1:20 and 1:80 respectively. He observed head, tail and mixed varieties of sperm agglutination. He demonstrated that sterility in these two men could very well be explained by the autoagglutination phenomenon, the agglutinating spermatozoa failing to penetrate the cervical mucus. Rumke (1954) described two instances of oligozoospermia in man, in whose semen sperm agglutinins could be demonstrated in high titre. Rumke and Hellinga (1959) reported the presence of sperm agglutinins
in the blood sera of approximately 3% of the male partner of sterile couples. They suggested that spermatostasis resulting from an occlusion in the vas deferens or the epididymis might lead to the formation of the autoantibody. Phadke and Padukone (1964) examined the blood sera from 50 azoospermic men with proved obstruction in the vas deferens for the presence of sperm agglutinins. They detected autoantibodies against spermatozoa in 13 of them. But they concluded that the presence of sperm agglutinins in the blood sera did not interfere with the fertility of the individual. Bratanov et al (1964) studied the formation of autoantibodies in breeding animals. Bulls, male goats, and male rabbits were autoimmunized with spermatozoal in suspension. Other groups, of these species were subjected to wounding of the testis or ligature of one or both vas deferens. No sperm agglutinins or precipitins were found prior to immunization treatment. But afterwards sera from all these animals had antibodies against their homologous antigens. Stevens et al (1965) assayed by haemagglutination technique, the presence of antibodies to human seminal plasma in blood sera of 618 men. They found in 30% of them a low antibody titre against human seminal plasma.

Sperm agglutination test:

This test was used for the first time for the detection of antispermatozoal antibodies by Landsteiner
(1899) and Metchnikoff (1899). Dittler (1920) employed this test to study the toxic effect of blood serum of the female rabbits immunized against homologous spermatozoa causing agglutination and immobilization of the same. McCartney (1923) detected spermatoxins in the vaginal and uterine secretions of immunized female rats, which had agglutinating effect upon the spermatozoa. Mudd and Mudd (1929) employed this test to study the species specificity of mammalian spermatozoa. Henle et al (1938) and Smith (1949) made use of this test to study the specific antigenicity of spermatozoa of bull and rabbits respectively. Kibrick et al (1952 a) developed a modified macroscopic agglutination test for the detection of sperm agglutinins and claimed that it gave over fourfold higher titres than the ordinary microscopic or macroscopic agglutination tests. Again Kibrick et al (1952 b) made an improvement in macroscopic sperm agglutination test by employing actively motile spermatozoa and a medium of increased viscosity containing 2.5 percent gelatin. They stated that this improved technique gave over one hundred-fold higher titre than ordinary sperm agglutination test. Nakabayashi et al (1961) made use of improved macroscopic agglutination test for detection of sperm agglutinin from blood serum of infertile human couples. Wilson (1954) and Lindahl and Hellinga (1959) found by sperm agglutination test auto-antibodies against spermatozoa in sterile men. Behrman and Otani (1963) tested the sera of immunized female guinea-
pigs against homologous testis by means of improved macroscopic sperm agglutination test of Kibrick et al (1952).

**Indirect haemagglutination test:**

Jones (1927) showed that antigens can be adsorbed on the surface of inert particles like collodion which can be agglutinated in the presence of specific antiserum. Extracts from a large number of bacteria had been absorbed onto respective antibacterial antibodies (Kabat and Mayer, 1961). Boyden (1951) observed that treatment of sheep erythrocytes with suitable concentration of Tannic Acid rendered them capable of adsorbing protein molecules and they were agglutinated in the presence of specific antisera. Stavitsky (1954) made some modifications in the test. Weil et al (1956) followed the technique of Landy and Trapani (1954) for detection of antispermatozoal antibodies in rabbits. Rao and Sadri (1960) used the highly sensitive haemagglutination technique of Boyden (1951) and Stavitsky (1964), for detection of small amounts of antispermatozoal antibodies. The slightly modified the technique in coating the erythrocytes with the antibody instead of antigen. Nakabayashi et al (1961) in their immunological investigations of serum from infertile persons employed Boyden's technique using supernatant fluid from saline washed, frozen and thawed sperm as antigen for coating the tannic-acid-treated sheep red blood cells.
Stevens *et al.* (1965) with the help of haemagglutination technique, assayed the blood sera of 618 patients for antibodies to human seminal plasma. They found a low antibody titre varying from 1:20 to 1:320 in 30% of sera tested. Behrman and Nakayama (1965) studied the antitestis antibodies in immunized rabbits by haemagglutination technique. They got high titre (1:1280) in animals immunized by intradermal and transvaginal methods. Weil and Roberts (1965) used haemagglutination technique for detection of circulating antibodies against seminal plasma. They found that 70% of the immunized female rabbits had antibody titre ranging from 1:40 to 1:320 as evidenced by haemagglutination test.

**Complement fixation test:**

Parsons and Hyde (1940) used this test for detection of antibodies against spermatozoa of bull, sheep, guinea pig, rat and rabbit. They claimed that the test was most reliable. Henle and Henle (1940) with the help of complement fixation test, detected antispermatozoal antibody in 60 to 70% of female guinea pigs immunized against homologous spermatozoa. Bull spermatozoa elicited antibodies in 100 percent guinea pigs, the titre ranging between 1:25 to 1:410. They used spermatozoal suspensions containing $5 \times 10^7$ cells per ml. as antigen in the test. Weil *et al.* (1956) used complement fixation test according to Kolmer's method with "2 full" units of complement to study the a
antigens of human seminal plasma. In their tests, rabbit immune sera, in serum dilutions varying between 1:80 and 1:640, reacted in complement fixation test with seminal plasma in dilution up to 1:1000000 or slightly more. The same sera also reacted with washed spermatozoa. One to four million spermatozoa were sufficient for complete inhibition of haemolysis with 0.01 to 0.0006 ml. of immune serum. Hunter and Hafs (1964) used complement fixation test to study the antigenicity and cross-reactions of bovine spermatozoa. They added serial dilutions of the antigen \((100 \times 10^7\) spermatozoa/ml.) to a 1:10 dilution of antiserum. The highest titre obtained by them in the complement fixation test was 1:2000.

**Precipitation test:**

The discovery and nomenclature of the precipitin reaction is attributed to Kraus (1897) who observed that a precipitate was formed when cell-free filtrates of broth cultures of typhoid bacillus were mixed with antityphoid serum. It has since been recognized that precipitating antibodies can be produced against most proteins and also against some polysaccharides. Precipitin reactions in gels added a new dimension to the study of antigen-antibody reactions.

Although antigen-antibody reaction in gels were reported as early as 1905 by Bechhold and were used by various investigators, their usefulness began with the work of Oudin (1946) who introduced the technique of single
diffusion. Subsequently Ouchterlony (1948) introduced the technique of double diffusion for the extensive study of precipitation reaction in gel. Farnum (1901) used precipitation test for the detection of specific precipitin against semen in female rabbits. Lewis (1941) demonstrated, by precipitation test, the antigenic similarity between alcoholic extracts of brain, testis and corpus luteum of bovine. Weil et al (1956) studied the antigens of human seminal plasma by precipitation test. They used this technique by layering undiluted immune serum beneath varying dilutions of antigen and then observing ring formation. They found that immune sera gave precipitation band with seminal plasma in dilutions up to 1:1000 or 1:10000. They also employed the technique of agar gel diffusion and observed three regions of precipitation. Rao and Sadri (1959) demonstrated by agar gel precipitation test, 15 to 16 antigens in human semen and seminal plasma. Again Rao and Sadri (1960) made use of the same technique for extensive study of the number of antigens present in buffalo semen. Hathaway and Hartree (1963) observed by agar gel diffusion technique, four precipitin lines between extracts of ram acrosomes and an anti-serum against these extracts. Hunter and Hafs (1964) used precipitation test in gel and found that rabbit ejaculated spermatozoa possessed seven antigens out of which five were shared with seminal plasma.

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MATERIALS AND METHODS
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Animals used:

During the course of present study various species of animals were used. For the preparation of antisera against buck semen, ten mature female goats of local non-descript type were procured. They were maintained under meticulous care and management and feedings. They were given adequate quantity of green grass and concentrate containing gram and ground nut cakes. They were also allowed for grazing in an enclosure for about 4 hours daily. Bucks maintained at Government Cattle Farm, Patna and at Semen Bank, Patna were used for collection of semen. Tharparkar bulls allotted under the progeny testing programme at the Government Cattle Farm, Patna and the bulls of Semen Bank, Patna were also used for collection of semen.

Investigations were extended to examine following groups of animals keeping normal as control:

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of animals examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infertile cows without apparent anatomical and/or physiological abnormalities of genitalia.</td>
<td>75</td>
</tr>
<tr>
<td>Infertile cows with anatomical and/or physiological abnormalities of genitalia.</td>
<td>50</td>
</tr>
<tr>
<td>Fertile cows, pregnant or recently calved (control).</td>
<td>15</td>
</tr>
<tr>
<td>Heifers. (control).</td>
<td>15</td>
</tr>
</tbody>
</table>
These cases were obtained from the Government Cattle Farm, Patna and from the Outdoor Clinic of the Gynaecology Department of Bihar Veterinary College, Patna. Only those cows which had history of 3 or more services without conception were included in these first two categories. Such cows were examined thoroughly per rectum and with the help of vaginal speculum to find out any definite anatomical or physiological abnormality that could be attributed for their failure of conception and were kept in the respective first two categories.

Materials collected for examination comprised blood sera from the infertile cows as "experimental" and that from healthy fertile cows and heifers of Government Cattle Farm, Patna as "control".

Preparation of antigens:

The following antigens were prepared and used in the present studies.

1. Antigen for hyper immunization.
2. Antigens for serological tests.
   (a) Whole semen.
   (b) Seminal plasma.
   (c) Freezed and thawed antigens.
      (i) Whole semen.
      (ii) Washed seminal spermatozoa.
      (iii) Epididymal spermatozoa.
1. Antigen for hyperimmunization:

Semen from five bucks of Semen Bank, Patna and 30 bucks of Government Cattle Farm, Patna was collected by means of Artificial vagina. Routine examination of freshly collected semen was done. The collection was made approximately twice in a week. Semen samples were pooled and merthiolate was added to a final concentration of 1:10000. It was stored in the deep freeze for further use.

2. Antigens for serological tests:

(a) Whole semen: Freshly collected semen was used for this purpose.

(b) Seminal plasma: Freshly collected and pooled semen was centrifuged at 2000 r.p.m. for 20 minutes. In order to protect the antigenic fraction due to heat, centrifugation was always done in cold room at 4°C. Clear seminal plasma was separated and stored in the deep freeze for use.

(c) Freezed and thawed antigens: Freezing and thawing of semen or spermatozoal suspension in saline was carried out with the object of obtaining more of the water soluble spermatozoal antigens.

(i) Whole Semen: Freshly collected and pooled semen.

(ii) Washed seminal spermatozoa: After separation of seminal plasma as in (b) spermatozoa were washed three times with normal saline and diluted to 3500 million sperms/ml with normal saline and stored
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(i) **Whole Semen** :- Freshly collected and pooled semen.

(ii) **Washed seminal spermatozoa** :- After separation of seminal plasma as in (b) spermatozoa were washed three times with normal saline and diluted to 3500 million sperms/ml. with normal saline and stored
in the deep freeze.

(iii) Epididymal spermatozoa:— Buck testicles together with the epididymis were obtained from the slaughter house soon after the animals were destroyed. They were brought to the laboratory in thermoflask. The capsule of the testis was removed and the testis along with epididymis and vas deferens was freed from blood as much as possible. With a 2 ml. syringe and needle normal saline was forced through the vas deferens. When the epididymis was distended by the fluid pressure, a slit like opening was made and the fluid containing sperm was collected in small glass container. The sperm count was made and a suspension containing 3500 million sperm/ml. was prepared and stored in the deep freeze.

The above mentioned three antigens viz. whole semen, washed seminal spermatozoa and epididymal spermatozoa were kept in the deep freeze at -20°C. Every alternate day the materials were removed from the deep freeze and kept for 30 minutes in the incubator at 37°C for thawing. The process of freezing and thawing was repeated for five times. Then the materials were centrifuged in cold room at 4°C. and the clear supernatent was separated and stored in the deep freeze after adding merthiolate 1 in 10,000.
Preparation of antisera:

Method of hyperimmunization:

Eight mature female goats were used for this purpose. Before the animals were subjected to immunization, normal blood sera and vaginal secretions were collected from each animal. These materials were kept in the deep freeze after addition of merthiolate to a final concentration of 1 in 10,000. Immunization was done by two methods viz.

(a) By parenteral route and
(b) By intravaginal route

(a) By parenteral route: Six goats were immunized by this method. The interval between the injections was of three days. The dilution of semen was made with normal saline. The schedule of immunization was as given below:

<table>
<thead>
<tr>
<th>Injections</th>
<th>Route</th>
<th>Dose (in ml.)</th>
<th>Dilution of semen</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>S/C</td>
<td>1.0</td>
<td>Neat.</td>
</tr>
<tr>
<td>Second</td>
<td>I/V</td>
<td>1.0</td>
<td>1:1</td>
</tr>
<tr>
<td>Third</td>
<td>I/V</td>
<td>2.0</td>
<td>1:1</td>
</tr>
<tr>
<td>Fourth</td>
<td>I/V</td>
<td>3.0</td>
<td>1:1</td>
</tr>
<tr>
<td>Fifth</td>
<td>I/V</td>
<td>4.0</td>
<td>1:1</td>
</tr>
<tr>
<td>Sixth</td>
<td>I/V</td>
<td>6.0</td>
<td>1:1</td>
</tr>
<tr>
<td>Seventh</td>
<td>I/V</td>
<td>8.0</td>
<td>1:1</td>
</tr>
</tbody>
</table>

S/C - Subcutaneous.
I/V - Intravenous.
Seven days after the last injection, each animal was bled from jugular vein and the serum was separated. Serum from each animal, after addition of Merthiolate 1 in 10,000 was distributed in different screw capped vials and stored in the deep freeze. Vaginal secretions were also collected at 7, 15 and 21 days after the last injection.

(b) **By intravaginal route**: Four goats, two new and two from the above group were immunized by intravaginal route. For this purpose a small sterile tampon was soaked with about two ml. of pooled buck whole semen containing only dead sperm. The vulva was cleared with sterile saline and the tampon soaked in antigen was inserted into the anterior part of vagina with the help of vaginal speculum. The tampon was allowed to remain there for 12 hours after which it was changed. A total of six applications were made in each goat.

After seven days of the last application, blood serum and vaginal secretions were obtained from each goat.

**Collection of vaginal secretion for detecting the presence of antibodies**: Vaginal secretion was collected from both groups of immunized goats. For this a small sterile cotton tampon slightly moistened with sterile normal saline was inserted deeply into the vagina with the help of a vaginal forceps to absorb the secretion. It was left there for about two hours and then was removed and immersed in five ml. of sterile saline in a test tube. The saline was stirred continuously for elution of absorbed secretion and the tampon was
pressed against the test tube wall and removed. Thus absorption and elution was performed for three times for each animal using the same tampon and saline. The solution was centrifuged at 2000 r.p.m. for 10 minutes to remove cell components and was stored. It was assumed that this supernatent represented a 1:5 dilution of vaginal secretion.

**Tests employed:**

The hyperimmune serum prepared against the whole semen was tested by following serological tests:

1. Sperm agglutination test
   - (a) Macroscopic test
   - (b) Microscopic test

2. Indirect Haemagglutination test

3. Complement fixation test

4. Precipitation test
   - (a) Ring test
   - (b) Gel diffusion test

Serum samples collected from repeat breeder or infertile cows of Government Cattle Farm, Patna and Outdoor Clinic of the Gynaecology department of Bihar Veterinary College, Patna as well as from healthy fertile control cows and heifers were put to sperm agglutination test only. Sperms from eight bulls stationed at Government Cattle Farm, Patna and Semen Bank, Patna were used as antigen in this test.
1. **Sperm agglutination test:**

   (a) **Macroscopic test:**

   The methods adopted for the Macroscopic sperm agglutination test was followed on the line of Kibrick et al (1952) with some modifications as follows:

   Immediately after collection of semen, sperm count was made and diluted to a concentration of 40 million spermatozoa per ml. An equal volume of gauze-filtered 10 percent gelatin in normal saline solution was added giving a sperm concentration of 20 million per ml. and a gelatin content of five percent. The gelatin solution and gelatin perm mixture were kept in a water bath at 37°C. to facilitate pipetting.

**Test:**

Serial dilutions of the inactivated sera to be tested were made with normal saline and 0.5 ml. of each dilution was kept in a presipitation tube. An equal volume of the spermatozoa-gelatin antigen was then added giving a final concentration of 10 million spermatozoa per ml. in 2.5 percent gelatin. The contents of the tubes were mixed thoroughly and incubated at 37°C. for two hours. Agglutination was evidenced by the appearance of white clumps. These were sought at each dilution in the immune sera tested before agglutination occurred in the lowest dilution of the inactivated normal serum.

The gelatin-prepared antigen plus diluent as
as well as known positive and negative sera comprised the "controls".

(b) Microscopic test:

Standard sperm suspension containing 40 million actively motile spermatozoa per ml. was prepared for the test. Two drops of inactivated serum and 1 drop of sperm suspension were mixed on a clean glass slide. A coverslip was put over the mixture and sealed by vaseline. The slide was examined under microscope at 30 minutes, 1 hour and two hours intervals for the evidence of sperm agglutination. Aggregation of 10 to 30 sperm cells per aggregate was taken as positive agglutination.

2. Indirect haemagglutination test:

The method of Boyden (1951) and Stavitsky (1954) was used with slight modifications.

Preparation of serum:— The serum was inactivated in a waterbath at 56°C. for 30 minutes. To remove heterophile agglutinins, 0.1 ml. of washed packed sheep erythrocytes was added to 1 ml. of serum and incubated in a waterbath for 1 hour with frequent shakings. The tube was centrifuged and clear supernatant serum was separated for use.

Preparation of erythrocytes: Blood from sheep was collected aseptically into an equal volume of modified Alsever’s solution. It was kept in the refrigerator for three days before use. When required, erythrocytes were washed thrice with N.S.S. at 2000 r.p.m. for 15 minutes.
Preparation of phosphate buffer saline (pH 7.2): An N/15 solution of Primary Potassium Phosphate was prepared containing 9.078 gms. of Potassium dihydrogen Phosphate (KH₂PO₄) in 1 liter of Distilled water.

An N/15 solution of secondary Sodium Phosphate was prepared containing 23.896 gms. of Disolium Hydrogen Phosphate (Na₂HPO₄·12H₂O) in 1 liter of Distilled water.

Both the solutions were sterilized by autoclaving at 15 lbs. pressure for 30 minutes. 30 ml. of Primary Potassium Phosphate solution were added to 70 ml. of secondary Sodium Phosphate solution to give a buffer of pH 7.2 and to it 0.85 gms. of Sodium Chloride was added.

Adsorption of antigen on tannic acid treated erythrocytes:

Equal volumes of 2.5% erythrocyte suspension and 1:10000 tannic acid in Phosphate buffer saline were mixed and incubated at 37°C in a waterbath for ten minutes. It was centrifuged, washed with Phosphate buffer saline and suspended to the original volume. These cells were kept in the refrigerator and used within 12 hours. The whole semen freeze-thawed antigen was diluted with Phosphate saline pH 6.4 to a concentration which would not spontaneously agglutinate tanned sheep cells. After 10 minutes at room temperature the cells were centrifuged, washed once with 1/100 normal rabbit serum and suspended in 1 ml. of 1/100 normal rabbit serum. A control sample of tanned cells was treated similarly except for the absence of the antigen to the adsorbed
Test:

Perspex plates having eight rows of ten cavities were used for doing this test. Phosphate buffer normal rabbit serum saline (Phosphate Buffer saline having pH 7.2, 99 parts and inactivated normal rabbit serum 1 part) was used as diluent for antiserum. The test was done according to the protocol given in Table I.

The whole system was left over night at room temperature and the results were taken next morning. The titre of the serum was its highest dilution showing complete agglutination of sensitized cells.

3. Complement fixation test:

In the present study complement fixation test was done according to the method of Kolmer (1951) with slight modifications.

Titration of haemolysin:—Haemolysin against sheep erythrocytes was obtained from Haffkine's Institute, Bombay. The titre of haemolysin used was the highest dilution showing complete lysis of sheep R.B.C. The titration was done according to the method of Prasad (1966) as shown in the protocol in Table II.

Preparation and titration of complement:—Sera collected from three to six healthy adult male guinea pigs were pooled and used as complement. The titre of the complement used was the highest dilution which showed complete haemolysis. The titration was done according to the method followed by Prasad (1966) as shown in Table III.
Preparation of sheep erythrocytes: - The blood was collected aseptically into an equal volume of modified Alsever's solution which was prepared as follows:

- Dextrose: 20.0 gms.
- Sodium Chloride: 4.20 "
- Sodium Citrate: 2.00 "
- Citric Acid: 0.55 "
- Distilled water: 1 liter.

The blood was kept in the refrigerator for three days before use. Whenever required the erythrocytes were washed thrice at 2000 r.p.m. for 15 minutes and finally suspended to make 4% suspension in normal saline.

Preparation of indicator system: - Equal volume of 4% sheep erythrocytes and haemolysin containing 16 M.H.D. per ml. were mixed together and incubated in a waterbath at 37°C. for 30 minutes so that 0.25 ml. of 2% sensitised cells contained 2 M.H.D. of haemolysin.

Preparation of serum: - The serum was inactivated in a waterbath at 56°C. for 30 minutes immediately before use.

Anti-complementary activity: - Anti-complementary activity of the antigen and antisera was found by the technique of Kolmer et al. (1951) as shown in the protocol in Table IV. Anticomplementary unit was the highest dilution showing slight inhibition of haemolysis at the end of secondary incubation at 37°C. for 30 minutes.

Complement fixation test: - The test was done according to the methods of Prasad (1966) as shown in the Protocol in Table V. The titre of the serum was the highest dilution showing complete fixation of two units of complement.
<table>
<thead>
<tr>
<th>Cavity No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer serum saline (pH 7.2) in ml.</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>Serum (1:5) in ml.</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>After mixing thoroughly 0.5 ml. of the contents from cavity No. 1 was added to cavity No. 2. The same process was repeated up to cavity No. 8 from which 0.5 ml. of the contents was discarded.</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution factor</td>
<td>1:10</td>
<td>1:20</td>
<td>1:40</td>
<td>1:80</td>
<td>1:160</td>
<td>1:320</td>
<td>1:640</td>
<td>1:1280</td>
<td>—</td>
<td>1:10</td>
</tr>
<tr>
<td>0.5% tanned and sensitised sheep erythrocytes in ml.</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.5% tanned unsensitised sheep erythrocytes in ml.</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Controls:**
- Cavity No. 9 = Tanned sensitised cells control
- Cavity No. 10 = Tanned unsensitised cells control.

The plate was kept overnight at room temperature.
### Table II

**Protocol for the titration of haemolysin**

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline (in ml.)</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
<td>0.9</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Haemolysin dilution (in ml.)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>(1:100) (1:200) (1:400) (1:800) (1:1600) (1:3200) (1:6400) (1:100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea pig complement (in ml.)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(1:20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% sheep erythrocyte suspension (in ml.)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Controls:**
- Tube No. 8 - Complement control.
- Tube No. 9 - Haemolysin control.
- Tube No. 10 - Normal saline control.

The system was incubated at 37°C for 30 minutes.
<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline (in ml.)</td>
<td>0.5</td>
<td>0.6</td>
<td>0.7</td>
<td>0.8</td>
<td>0.8</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Guinea pig complement (1:20)</td>
<td>0.5</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>25 sensitised sheep erythrocytes containing 4 M.H.D. of Haemolysin (in ml.)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>25 unsensitised sheep erythrocytes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

**Controls**
- Tube No. 6: Complement control.
- Tube No. 7: Haemolysin control.

The system was incubated in water bath at 37°C for 30 minutes.
<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen or serum undiluted (0.25 ml.)</td>
<td></td>
<td>1:2</td>
<td>1:4</td>
<td>1:8</td>
<td>1:16</td>
<td>1:32</td>
<td>1:64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal saline.</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.75</td>
</tr>
<tr>
<td>Complement 2 units (in ml.)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>The system was incubated at 37°C in water bath for 30 minutes.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% sensitised cells containing 2 units of haemolysin (in ml.)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>2% unsensitized cells (in ml.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>The system was incubated in water bath at 37°C for 30 minutes.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Controls**
- Tube No. 8 - Haemolysin control
- Tube No. 9 - R.S.C. Control.
<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline (in ml.)</td>
<td>0.40</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.80</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Serum (in ml.)</td>
<td>0.10</td>
<td>After mixing 0.25 ml of contents from tube No.1 was serially transferred upto tube No.7 from which 0.25 ml. was discarded. 0.25</td>
<td>0.25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dilution factor.</td>
<td>1:5</td>
<td>1:10</td>
<td>1:20</td>
<td>1:40</td>
<td>1:80</td>
<td>1:160</td>
<td>1:320</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Antigen containing 4 antigenic units (in ml.)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>-</td>
<td>-</td>
<td>0.25</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>Complement 2 units (in ml.)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>-</td>
<td>0.25</td>
<td>-</td>
<td>0.25</td>
</tr>
</tbody>
</table>

The system was incubated in water bath at 37°C for 30 minutes.

2% sensitized cells containing 2 M.H.D. 0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.25 The system was incubated in water bath at 37°C for 30 minutes.

Controls: - Tube No. 8 & 9 - Serum controls.
- Tube No. 10 & 11 - Antigen controls.
- Tube No. 12 - Normal saline control.

* - Serum of lowest dilution was used.
4. **Precipitation test** :

(a) **Ring test** :

Supernatant of the freeze-thawed whole semen was used as antigen for this test. The hyper immune serum was diluted serially with normal saline and 0.5 ml. of the diluted serum was placed in precipitation tubes. With the help of a well drawn out Pasteur pipette, 0.5 ml. of the antigen was layered over the serum samples at different dilutions and only those tubes were observed further where the serum and the antigen formed distinct layers. The result was read at 30 and 60 minutes at room temperature. Further readings were made after incubation at 37°C for two hours and finally standing in the refrigerator overnight.

For controls, tubes containing saline alone, saline with serum from healthy and immunized animals and saline with antigen were used.

Parallel set of precipitation tubes, where the antigen was diluted serially and placed in 0.5 ml. aliquots layered with 0.5 ml. of the serum samples, was also prepared.

The formation of an opalescent ring at the antigen-serum interphase read against a black background was noted as a positive reaction.

(b) **Gal test** :

The technique employed for the preparation of agar gel was on the line of Ouchterlony (1945) with slight
modifications.

Preparation of agar gel: "Difco" Agar powder was mixed with sufficient quantity of glass distilled neutral water. After sometime when the agar settled at the bottom, the supernatant was decanted and fresh distilled water was poured over the agar. The process was repeated for ten times. Finally the agar was poured in a large size Petridish and kept in an incubator for drying. From the washed and dried agar, Agar gel having the following composition was prepared:

Agar 1.5 gms.
Phosphate Buffer saline-100 ml.

It was autoclaved at 10 lbs. pressure for ten minutes and merthiolate solution was added to a final concentration of 1 in 10,000. 25.0 ml. of molten agar was poured into clean "Pyrex" Petridishes of about four inches diameter. With the help of pipette, about 4.0 ml. of molten agar was also put on the clean dry slide to give a thin uniform film.

Procedure: After the agar had set, wells were cut with help of a glass tube of 8 mm. diameter by keeping the plate or slide over a pattern made on a paper. The cut pieces of agar were removed by means of a needle and the bottom of each well was sealed with drop of molten agar. Usually one cup was placed in the centre and two or six others in circle. With the help of fine Pasteur-pipettes, the central cup was filled with antiserum and the surrounding cups with different antigens. The charged plates and slides were kept under the
cover of Bell Jar at room temperature An open Petridish containing cotton soaked in water was placed under the Bell Jar to prevent evaporation of the reacting fluids. The plates were examined daily and the development of precipitation lines was noted.

"*******
**
RESULTS
RESULTS

Hyperimmunization: In order to study the antigenicity of semen, six goats were hyperimmunized as per the schedule given in the previous chapter. All goats during immunization showed anaphylactic reactions characterised by coughing, dyspnoea, tremors, and frequent micturition and defaecation. These reactions developed in third and subsequent injections. Three goats actually fell down after receiving the injections of semen. After 1-2 hours of injection, a rise in temperature of all goats was observed.

SPERM AGGLUTINATION TEST:

Macroscopic test:

In order to ascertain whether sperm agglutinins were present in sera of normal healthy female goats, they were subjected to sperm agglutination test. Normal sera from all the eight goats showed the sperm agglutination titre below 1:5 except one goat no.3 which agglutinated sperm in 1:5 dilution. Six of these goats were immunized by parenteral route with homologous whole semen. Sera collected from these immunized goats at various intervals were tested for the presence of sperm agglutinins. The details of the results of sperm agglutination test are given in Table 1.

Significant rise in titre was observed in all immunized goats. The maximum titre was found to be 1:2560 in four goats. The lowest titre was 1:640 in one goat no.6. Out of the two sera from healthy control goats only one, in undiluted form, could agglutinate the sperm.
TABLE NO. 1

Results of sperm agglutination test with sera of goats immunized by parenteral route.

<table>
<thead>
<tr>
<th>No. of Immunized animals</th>
<th>Undiluted control</th>
<th>Reciprocal of serum titre.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>G 1 Immunized.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G 2</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 3</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 4</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 5</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 6</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 7 Control.</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G 8</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

Notes: + Complete agglutination.  
† Doubtful.  
- No agglutination.
Cross-reactions in sperm agglutination test:

In order to study the cross-reactions between buck and bull spermatozoa, hyperimmune serum prepared against whole semen of buck was tested for the presence of sperm agglutinins against buck spermatozoa and bull spermatozoa. The results are shown in Table 2.

**TABLE No.2**

Sperm agglutination test with antisera prepared against buck semen.

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Immunized or control</th>
<th>Reciprocal of serum titre with Buck spermatozoa</th>
<th>Bull spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>G 1</td>
<td>Immunized</td>
<td>2560</td>
<td>80</td>
</tr>
<tr>
<td>G 2</td>
<td>&quot;</td>
<td>2560</td>
<td>160</td>
</tr>
<tr>
<td>G 3</td>
<td>&quot;</td>
<td>2560</td>
<td>80</td>
</tr>
<tr>
<td>G 4</td>
<td>&quot;</td>
<td>1280</td>
<td>40</td>
</tr>
<tr>
<td>G 5</td>
<td>&quot;</td>
<td>2560</td>
<td>40</td>
</tr>
<tr>
<td>G 6</td>
<td>&quot;</td>
<td>640</td>
<td>40</td>
</tr>
<tr>
<td>G 7</td>
<td>Control.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G 8</td>
<td>&quot;</td>
<td>N</td>
<td>-</td>
</tr>
</tbody>
</table>

N = Agglutination in undiluted serum.

It is apparent from the Table that all six hyperimmune sera, in addition to agglutinating the homologous spermatozoa, also agglutinated heterologous (bull) spermatozoa in low titres.
Sperm agglutinin-absorption test:

Hyper-immune-serum from goat no. 1 was absorbed with homologous and heterologous spermatozoa. The absorbed antiserum was tested for the presence of sperm agglutinins. The results are shown in Table 3.

**TABLE NO.3**

Sperm agglutinin - absorption test with serum from goat no. 1.

<table>
<thead>
<tr>
<th>Semen absorbed with</th>
<th>Reciprocal of serum titre before absorption</th>
<th>Reciprocal of serum titre after absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buck spermatozoa</td>
<td>Bull spermatozoa</td>
</tr>
<tr>
<td></td>
<td>2660</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>2660</td>
<td>80</td>
</tr>
</tbody>
</table>

It was seen that when buck spermatozoa was used for absorption it removed the sperm agglutinins against homologous germ cells and against the spermatozoa of bulls as well. But, when bull spermatozoa was used for absorption, the agglutinins against them were completely absorbed but the absorbed serum still contained agglutinins against the buck spermatozoa in significant titre.

**Microscopic test:**

Three types of agglutination were seen in the early stage: (a) head to head (b) tail to tail and (c) mixed
or head to tail. Within a few minutes, however, the clumps of still actively motile spermatozoa became entangled with other clumps and with individual spermatozoa (Plate-II). After 3-6 minutes the clumps were quite large and continued to grow as long as some spermatozoa remained motile. All the large clumps had a similar appearance, which was predominantly that of "tail" agglutination, with free sperm heads visible at the periphery of the clumps.

**Examination of vaginal secretion for the presence of antibodies:**

Vaginal secretions were collected from six goats before and at different intervals after immunization. The supernatant fluid of the secretions were put to sperm agglutination test to analyse the level of antibodies. The results are summarised in Table 4 and Graph 1.

**Table No. 4.**

Antibodies in the vaginal secretion of the immunized goats.

<table>
<thead>
<tr>
<th>No. of goats</th>
<th>Reciprocal of sperm agglutination titre of vaginal secretions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>G 1</td>
<td>-</td>
</tr>
<tr>
<td>G 2</td>
<td>5</td>
</tr>
<tr>
<td>G 3</td>
<td>-</td>
</tr>
<tr>
<td>G 4</td>
<td>-</td>
</tr>
<tr>
<td>G 5</td>
<td>5</td>
</tr>
<tr>
<td>G 6</td>
<td>5</td>
</tr>
</tbody>
</table>
GRAPH NO. 1

Showing Antibody Titre In Vaginal Secretion

LEGEND
- GOAT NO. 3
- GOAT NO. 2
- GOAT NO. 1

Antibody Titre

Time Interval

Before Immunisation
One Week After Immunisation
Two Weeks After Immunisation
Three Weeks After Immunisation
GRAPH NO.II

Showing Antibody Titre In
Vaginal Secretion

LEGEND
- GOAT NO.4
- GOAT NO.5
- GOAT NO.6

ANTIBODY TITRE

TIME INTERVAL

BEFORE IMMUNISATION ONE WEEK AFTER IMMUNISATION TWO WEEKS AFTER IMMUNISATION THREE WEEKS AFTER IMMUNISATION
Significant rise in antibody titre in the vaginal secretion was observed in four out of six immunized goats three weeks after the hyperimmunization. However, rising tendency of the antibodies with the lapse of time after hyper immunization was not a regular feature.

**INDIRECT HAEMAGGLUTINATION TEST:**

The results of indirect haemagglutination test with the sera of six parenterally immunized goats with buck semen are given in Table 5. The supernatant fluid after centrifuging the freeze-thawed whole semen of buck was used as antigen in this test.

Out of six hyperimmunized goats, the sera of five animals showed highest titre upto $1:640$ and of one showed titre upto $1:320$. Sera from control animals showed titres only upto $1:10$ dilution.

**COMPLEMENT FIXATION TEST:**

In order to study the response of complement fixing antibodies in hyperimmunized goats, complement fixation test was employed. Sera of six hyperimmunized and two control goats were put to this test using supernatant fluid obtained after centrifuging freeze-thawed whole buck semen as antigen. The results of the tests are presented in Table 6.

It will be apparent from the Table 6 that out
of six hyperimmunized goats, two gave a titre of 1:160, one upto 1:80 and the rest three upto 1:20.

**COMPARISON OF SPERM AGGLUTINATION, INDIRECT HAEMAGGLUTINATION & COMPLEMENT FIXATION TESTS:**

The titres obtained with sperm agglutination, indirect haemagglutination and complement fixation tests have been summarised in Table 7.

It will be apparent from the Table 7 that higher titres were found with sperm agglutination than haemagglutination and complement fixation tests. It was also found that haemagglutination test gave better titres than complement fixation test.

**PRECIPITATION TEST INagar gel:**

In the present study, gel-diffusion test was carried out only by double diffusion technique. For this purpose various antigens such as freeze-thawed whole-semen, seminal plasma, freeze-thawed ejaculated spermatozoa and epididymal spermatozoa were used. In order to analyse various precipitins present, the hyper-immune sera prepared in the goats were employed in the test. Results of double diffusion test in which antiserum to whole buck semen was used, are presented in Table 8.
<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Immunized or control</th>
<th>Reciprocal of serum titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>G 1</td>
<td>Immunized</td>
<td>+</td>
</tr>
<tr>
<td>G 2</td>
<td>&quot;</td>
<td>+</td>
</tr>
<tr>
<td>G 3</td>
<td>&quot;</td>
<td>+</td>
</tr>
<tr>
<td>G 4</td>
<td>&quot;</td>
<td>+</td>
</tr>
<tr>
<td>G 5</td>
<td>&quot;</td>
<td>+</td>
</tr>
<tr>
<td>G 6</td>
<td>&quot;</td>
<td>+</td>
</tr>
<tr>
<td>G 7</td>
<td>Control</td>
<td>+</td>
</tr>
<tr>
<td>G 8</td>
<td>&quot;</td>
<td>+</td>
</tr>
</tbody>
</table>

Note - + positive,  
† Positive,  
- Negative.
<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Immunized or control</th>
<th>Reciprocal of serum titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>G 1 Immunized</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 2</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 3</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 4</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 5</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 6</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G 7 Control</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>G 8</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

**Note:**
- + No haemolysis.
- † Incomplete haemolysis.
- Complete haemolysis.
<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Immunized</th>
<th>Reciprocal titre of sperm agglutination</th>
<th>Reciprocal titre of indirect haemagglutination</th>
<th>Reciprocal titre of complement fixation</th>
<th>Agglutination in undiluted serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>250</td>
<td>640</td>
<td>640</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>G2</td>
<td>250</td>
<td>640</td>
<td>640</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>G3</td>
<td>320</td>
<td>640</td>
<td>640</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>G4</td>
<td>320</td>
<td>640</td>
<td>640</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>G5</td>
<td>320</td>
<td>640</td>
<td>640</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>G6</td>
<td>400</td>
<td>640</td>
<td>640</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>G7</td>
<td>400</td>
<td>640</td>
<td>640</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>G8</td>
<td>400</td>
<td>640</td>
<td>640</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

M - Agglutination in undiluted serum.
TABLE NO. 8.

Results of agar gel diffusion test using hyper immune serum against buck semen.

<table>
<thead>
<tr>
<th>Antigens used</th>
<th>No. of precipitation lines formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moderate lines</td>
</tr>
<tr>
<td>Freeze-thawed semen.</td>
<td>4</td>
</tr>
<tr>
<td>Seminal plasma.</td>
<td>2</td>
</tr>
<tr>
<td>Freeze-thawed washed spermatozoa.</td>
<td>-</td>
</tr>
<tr>
<td>Freeze-thawed epididymal spermatozoa.</td>
<td>-</td>
</tr>
</tbody>
</table>

The antisera prepared against whole buck semen gave at least ten lines with freeze-thawed semen, nine lines with seminal plasma, five lines with freeze-thawed ejaculated spermatozoa and four lines with freeze-thawed epididymal spermatozoa (Plate - III). All precipitin lines could not be depicted photographically.

To prove that spermatozoa have some antigens different from seminal plasma, the inhibition test was carried out. Equal amounts of hyperimmune serum to whole semen and seminal plasma were mixed. The mixture was incubated at 37°C for 30 minutes to neutralize the antibodies to seminal plasma antigens. Again the gel diffusion test was performed with this absorbed antiserum and different antigens, the results of which are summarised
in the Table 9.

**TABLE NO. 9.**

Agar gel diffusion test using antiserum to buck semen absorbed by seminal plasma.

<table>
<thead>
<tr>
<th>Antigens used</th>
<th>No. of precipitin lines formed.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>lines.</td>
</tr>
<tr>
<td>Freeze-thawed semen.</td>
<td>1</td>
</tr>
<tr>
<td>Seminal plasma.</td>
<td>-</td>
</tr>
<tr>
<td>Freeze-thawed washed spermatozoa.</td>
<td>1</td>
</tr>
<tr>
<td>Freeze-thawed epididymal spermatozoa.</td>
<td>-</td>
</tr>
</tbody>
</table>

Absorption of hyperimmune serum with seminal plasma revealed that epididymal and washed ejaculated spermatozoa possessed two sperm-specific antigens which were not common to seminal plasma (Plate - IV). Seminal plasma did not show any precipitin lines with absorbed antiserum whereas it had shown nine lines with unabsorbed antiserum.

**Transvaginal immunization:**

Two goats which were previously immunized against buck semen by parenteral route were included in this immunization, along with two fresh goats. After 45 days of the immunization by parenteral route, serum
samples were collected from two immunized goats. These serum samples were tested for the level of antibodies by sperm agglutination and indirect haemagglutination test. The results of the test are given in the Table 10.

**Table No. 10.**

Results of haemagglutination and sperm agglutination test with sera collected after seven days & 45 days of parenteral immunization.

<table>
<thead>
<tr>
<th>No. of goats</th>
<th>Reciprocal of titre after seven days.</th>
<th>Reciprocal of titre after 45 days.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G 3</td>
<td>640</td>
<td>2550</td>
</tr>
<tr>
<td>G 4</td>
<td>320</td>
<td>1260</td>
</tr>
</tbody>
</table>

It is apparent from the Table that 45 days after immunization the haemagglutination titre and spermagglutination titre had appreciably reduced. At this time a sterile cotton tampon soaked in 2 ml. of buck whole semen was instilled into the vagina. Tampon was changed at the interval of 12 hours. This instillation was continued for three days.

Two fresh female goats were hyperimmunised by this method of transvaginal immunization. After seven days of this treatment serum-samples were collected from all the four goats. In order to determine any response in the level of antibodies, serum samples were put to
indirect haemagglutination and sperm agglutination tests. The results of the tests are shown in Table 11.

**TABLE NO. 11.**

Results showing serum titre before and after trans-vaginal immunization.

<table>
<thead>
<tr>
<th>Goat no.</th>
<th>Immunized &amp; control.</th>
<th>Reciprocal of titre before instillation of semen into vagina</th>
<th>Reciprocal of titre after instillation of semen into the vagina.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G 3</td>
<td>Immunised by parenteral route.</td>
<td>40</td>
<td>160</td>
</tr>
<tr>
<td>G 4</td>
<td>20</td>
<td>40</td>
<td>160</td>
</tr>
<tr>
<td>G 9</td>
<td>Non-immunised fresh goats.</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>G 10</td>
<td>10</td>
<td>N</td>
<td>10</td>
</tr>
<tr>
<td>G 7</td>
<td>Control.</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>G 8</td>
<td>&quot;</td>
<td>10</td>
<td>N</td>
</tr>
</tbody>
</table>

N = Agglutination in undiluted serum.

In goats number 3 and 4 which had been previously immunized by parenteral route, there was a remarkable increase in titre by instillation of semen into the vagina as evidenced by sperm agglutination and indirect haemagglutination test.

None of the fresh animals (9 & 10) showed any increase in antibody titre even after instillation of semen into their vagina.
Goat no. 7 and 8 were not subjected to intillation of semen and they did not show any titre with spermagglutination or indirect haemagglutination test.

**Examination of vaginal secretion for the presence of antibodies:**

Vaginal secretions were collected from four goats after seven days of intravaginal instillation of semen. They were centrifuged to remove the cell-components and subjected to sperm agglutination and indirect haemagglutination tests, results of which are shown in Table 12. Vaginal secretions had no haemagglutinating antibodies before or after intravaginal instillation of semen. Sperm agglutinins were present in low titre in the vaginal secretion before instillation of semen and the same low titres were maintained after instillation except in one animal in which there was slight increase in titre.

**EFFECT OF HYPERIMMUNIZATION WITH BUCK SEMEN ON FERTILITY IN EXPERIMENTAL FEMALE GOATS:**

In order to study whether experimental goats immunized against homologous semen, conceive or not, they were kept under observation. All of them showed the signs of oestrous at regular intervals. They were artificially inseminated with buck semen when in oestrus. Later on they were kept along with two mature bucks for natural mating. Time to time the goats were examined for pregnancy. After three months of their immunization four of them were
<table>
<thead>
<tr>
<th>No. of Gates</th>
<th>Rectified after 1st Inst.</th>
<th>Rectified before 1st Inst.</th>
<th>Test of semen into vagina.</th>
<th>Test of semen into vagina.</th>
<th>Indirect haemagglutination</th>
<th>Indirect haemagglutination</th>
<th>Sperm</th>
<th>Sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

**Method**

Screening of Gates Immunized by Transfusion

Results of sperm agglutination and indirect haemagglutination test with vaccine

**Table W°C 12**
sacrificed and their genitalia examined for the presence of embryo. On post-mortem examination it was found that their ovaries were functioning normally but there was no sign of pregnancy. During the course of observation two goats died due to accidental death.

Two healthy controls were also kept along with mature bucks and pregnancy was observed in one of them whereas the other remained infertile even after three months.

**STUDIES OF SERUM SAMPLES FROM INFERTILE COWS BY SPERM AGGLUTINATION TEST:**

A total of 140 cows of Government Cattle Farm, Patna and those brought to the Outdoor Clinic of Gynaecology Section were examined thoroughly and according to the condition of their genitalia they were classified into three categories. Serum samples were collected from all groups of animals. Besides, serum samples were also collected from 15 heifers.

Serum samples collected from these animals were put to sperm agglutination test. Semen from eight Tharparker bulls were used in the sperm agglutination tests as antigen.

Results of sperm agglutination test done with the sera of different categories of animals are detailed in Table 13, 14, 15, 16 and 17.
**TABLE No. 13.**

Results showing reciprocal of the sperm agglutination titre of sera of infertile cows without any apparent abnormalities.

<table>
<thead>
<tr>
<th>RECIPROCAL OF TITRE</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>160</th>
<th>320</th>
<th>640</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of reactors.</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>10</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>% of reactors.</td>
<td>5.3%</td>
<td>8%</td>
<td>6.6%</td>
<td>13.3%</td>
<td>21.3%</td>
<td>21.3%</td>
<td>24%</td>
<td></td>
</tr>
<tr>
<td>% of non-specific reactors.</td>
<td>20.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of specific reactors.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE No. 14.**

Results showing reciprocal of the sperm agglutination titre of sera of infertile cows with apparent abnormalities.

<table>
<thead>
<tr>
<th>RECIPROCAL OF TITRES</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>160</th>
<th>320</th>
<th>640</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of reactors.</td>
<td>20</td>
<td>15</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>% of reactors.</td>
<td>40%</td>
<td>30%</td>
<td>16%</td>
<td>6%</td>
<td>4%</td>
<td>2%</td>
<td>-</td>
<td>2%</td>
</tr>
<tr>
<td>% of non-specific reactors.</td>
<td>92.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of specific reactors.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.0%</td>
</tr>
</tbody>
</table>
### Table No. 15

Results showing reciprocal of the sperm agglutination titre of sera of fertile cows (control).

<table>
<thead>
<tr>
<th>Reciprocal of Titres</th>
<th>N 5</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>160</th>
<th>320</th>
<th>640</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of reactors.</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of reactors.</td>
<td>46.6% 20% 26.6% 6.6%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of non-specific reactors.</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of specific reactors.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nil.</td>
</tr>
</tbody>
</table>

### Table No. 16

Results showing reciprocal of the sperm agglutination titre of sera of heifers (control).

<table>
<thead>
<tr>
<th>Reciprocal of Titres</th>
<th>N 5</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>160</th>
<th>320</th>
<th>640</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of reactors.</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of reactors.</td>
<td>66.7% 20.0% 13.3%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of non-specific reactors.</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of specific reactors.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nil.</td>
</tr>
</tbody>
</table>
It will be apparent from Table 13 that out of 75 sera from infertile cows without any apparent abnormality, four gave titre upto 1:5, 6 upto 1:10, 5 upto 1:20, 10 upto 1:40, 16 upto 1:80, 16 upto 1:160 and 18 upto 1:320. It was found that 20% of the animals gave non-specific reactions (upto 1:20) and 80% gave specific reactions (above 1:20). The highest titre obtained was 1:320.

Out of 50 sera from infertile cows with apparent abnormalities, 20 gave titre in undiluted sera, 15 gave, titre upto 1:5, 8 upto 1:10, 3 upto 1:20, 2 upto 1:40, and 1 upto 1:80 and only one upto 1:320. It was found that 92% of the animals gave non-specific reactions (upto 1:20) and 8% gave specific reactions (above 1:20).

Out of 15 sera from healthy fertile cows (control), 7 caused agglutination only in undiluted form, 3 gave titre upto 1:5, 4 gave titre upto 1:10 and one upto 1:20. It was found that all sera from fertile control cows gave only non-specific reaction (upto 1:20).

Out of 15 sera from heifers (control), ten showed agglutination in undiluted form, 3 gave titre upto 1:5 and 2 upto 1:10. It showed that heifers which have not been covered by any bull did not carry in their sera any specific agglutinin against the bull sperm.
<table>
<thead>
<tr>
<th>Sera.</th>
<th>No.</th>
<th>Negative</th>
<th>Positive</th>
<th>Percentage of positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infertile cows without any apparent abnormalities.</td>
<td>75</td>
<td>15</td>
<td>26</td>
<td>34</td>
</tr>
<tr>
<td>Infertile cows with apparent abnormalities.</td>
<td>60</td>
<td>46</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>Fertile cows (control).</td>
<td>15</td>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heifers (control).</td>
<td>15</td>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
DISCUSSION

In order to gauge the antigenicity of each of the pure strains, they were administered to sensitized guinea pigs. The results generally showed no serious anaphylactic reactions. In some instances, however, the results were not entirely clear. The results varied from one animal to another, with some showing a strong reaction, while others showed none at all.

One interesting observation was the length of time required for the appearance of the antibodies. In some cases, antibodies were detected within hours of the injection, while in others, it took several days. This variability is important to consider when studying the immune response to antigens.
In order to study the antigenicity of buck semen six goats were hyperimmunized by parenteral route. During hyperimmunization anaphylactic reaction was observed in all the goats generally in 3rd and subsequent intravenous injections. No such reaction in goats has been described previously however, Kiddy et al (1959) observed a similar reaction in heifers while injecting bull semen into them, but they failed to demonstrate any correlation between the reaction and antibody titre which was a definite observation in the experiments described here.

This anaphylaxis was a manifestation of the combination of antigen with antibody in vivo, but it indicated only gross correlations between the levels of circulating antibody and the degree of anaphylactic sensitivity. These physical reactions indicated that an immunological response had been obtained and this was later confirmed by demonstrating high titres of specific antibodies in the sera by the different serological techniques.

Castano (1926) could not observe anaphylactic reactions in any instances while giving subcutaneous injections of semen into guinea pigs. However, much larger amounts of antigen are required in subcutaneous injections to produce anaphylaxis and also the development of symptoms is delayed. These might be responsible for Castano's failure.
to observe this reaction in the case of guinea pigs.

The correlation between the anaphylactic reactions and the high titre of antibody shown by the same goat appeared to be quite significant and it indicated that the animals which appeared to be more prone to the anaphylactic reaction were also sensitive to an appreciable extent in showing a response of high antibody in them.

The buck semen on parenteral inoculation in female goats was observed to induce antibody formation in appreciably high titres (1:2560) as evidenced by sperm agglutination test. This gave an indication that buck semen is antigenic and can give rise to the formation of sperm agglutinins in the sera of the females of homologous species.

The antigenic properties of the mammalian spermatozoa shown by Smith (1949) and the immunization of heterologous hosts with bull spermatozoa (Docon et al., 1952) and with bull and ram spermatozoa (Baum et al., 1959; Cvellars, 1965) are in confirmation with the present findings, supporting the antigenicity of semen. Immunization with semen of homologous species has been observed in rats (McCarty, 1923; Fogelson, 1926), rabbits (Guyer, 1922; Dittler, 1926; Mudd and Mudd 1929; Behrman and Nakayama, 1965) and bulls (Menge et al., 1962) and was also observed in bucks in the present study.

It was observed that antisera prepared against buck semen reacted strongly with buck spermatozoa showing
species-specific antigenic character. The results are quite in agreement with the findings of Hektone and Manly (1923), Mudd and Mudd (1929), Edward et al. (1964) and Cvellar (1965). Supporting this are the findings of Bennette (1965) also who observed species-specificity in seminal plasma proteins of bull, ram, boar and rabbit by agar gel electrophoresis.

Observations regarding the cross reaction test in which the antibody produced against buck semen showed a significant agglutinin titre (1:160) also with the bull spermatozoa elucidates that there exists antigenic similarity between the buck and bull spermatozoa. Similar cross reactions were noted between spermatozoa of bull and ram (Mudd & Mudd, 1929; Matousek, 1964; Henle, 1938) and between ram and rabbit (Pomeranke, 1928), while Cvellar (1965) could not find any cross reaction in bovine and ovine spermatozoa. Since the relationship between buck and bull spermatozoa is unknown, this finding records the first observation of such relationship.

This observation points to the existence of a similarity in the antigenic structure of the sperm. It seems that there may be some fraction in the antigenic component of both sperm which are common and this observation further demands the extraction of those fraction which are similar and dis-similar so that the importance of spermatozoa playing a significant role, in the causation of derrangement of the normal reproductive phenomenon can
be explained on more rational and authentic manner.

To study further the specificity of cross reaction, absorption of anti sera against buck semen by buck and bull spermatozoa was performed. When antisera was observed with buck spermatozoa, it was noticed that it absorbed all the antibodies against buck spermatozoa as well as against bull spermatozoa, but when bull sperms were used for absorption they failed to remove all antibodies against buck spermatozoa, though they removed all antibodies against bull sperm. This indicates that both the antigens are not completely identical, they only share some antigenic fraction.

The results obtained in the present study on absorption of antisera agree well with the findings of Matousek (1964) on antisera against bull spermatozoa. It appears that immunization with spermatozoa caused the production of antibodies against antigens that are common to all spermatozoa of whatever species, and the antigens are located, as suggested by Smith (1949) in different layers, or at different depths, in the spermatozoa of different species.

Observations regarding, the types of agglutination of spermatozoa in the microscopic agglutination test revealed that tail to tail agglutination was dominantly found with hyperimmune sera whereas head to head agglutination was seen even with normal control sera. It may be concluded from the above finding that tail to tail
agglutination is of specific type of agglutination whereas head to head type is of non specific nature. Almost similar reports were made by Smith (1949) and Beck et al (1962) who demonstrated this, by microscopic agglutination and immunofluorescent antibody technique respectively. The present results are quite in agreement with the view of Kiddy et al (1959) who reported immune sera of heifers immunized with rabbit sperm causing tail to tail agglutination of rabbit sperm in contrast to head to head type produced by normal sera. However, there are controversial reports like those of Rumke and Hellinga (1959) who observed all types of agglutination and Behrman and Otani (1963 a) who found mainly head to head type of sperm agglutination with the sera of immunized guinea pigs. It appears that the type of specific agglutination varies with the species and no generalisation can be made.

In the present study an attempt was made to determine if anti-sperm antibodies do appear in the vaginal secretions and agglutinate the sperm. Taking into account of the results of sperm agglutination test summarised in Table 4, it seems that there exists some relationship between the circulating and local antibody as all the hyperimmunized goats had antibodies in demonstrable titres in the vaginal secretions, 3 weeks after immunization. In 4 out of six goats the rising tendency of the antibody titres with the lapse of time was markedly evident. Since the goats were immunized exclusively through the parenteral
route and were not allowed to come in contact with any buck, the presence of antibody in the vaginal secretions was, probably, due to their escape and excretion from the circulation into the reproductive tract. Thus it may be concluded that the induced sperm agglutinins are capable of passing from the circulation into the genital tract in female goats.

With the available literatures it seems that such escape and excretion of circulating antibody has not been described previously in case of goats, but has been observed in guinea pigs (Parsons and Hyde, 1940; Ashitaka et al., 1964), rats (McCartney, 1923) and rabbits (Pomeranke, 1928; Garcia Alfonso, 1959). Edwards (1960), however, contradicts this observation in case of rabbits.

Freezing and thawing of the whole semen released the antigen which was absorbed on the surface of R. B. C. as observed during the indirect haemagglutination test. However, it appears that the antibodies titres against such an antigen was not as high as against the spermatozoa. Whether the soluble antigens released as a result of freezing and thawing or components of whole semen other than sperm were taking part in this system, could not be ascertained. Weil et al. (1956) were the first to use indirect haemagglutination test for detection of antibodies in rabbits against human seminal plasma. The highest titre (1:640) of the indirect haemagglutination test in the present investigation is very low in comparison to the titre of 1:10000 obtained.
by Weil et al (1956). Again Weil et al (1965) obtained very low titres 1:40 to 1:320 in indirect haemagglutination test with the sera of rabbits immunized against human seminal plasma. However, the highest titres obtained in the present investigation is well comparable with titres (1:1280) obtained in indirect haemagglutination test by Behrman and Nakayama (1965).

The level of antibodies formed by active immunization of female goats with buck semen was demonstrated by complement fixation test. The complement fixation test is considered as one of the most sensitive in most of the bacterial antigen-antibody reactions. Parsons and Hyde (1940) also claimed it to be most sensitive serological tool for the detection of seminal antibodies. But in the present investigation this test has given very poor response as the highest titre obtained was only 1:160. The opinion of the previous workers as reviewed, regarding the sensitivity of the complement fixation test in the seminal antibodies, will need to be substantiated by further investigation on large number of cases using different types of antigens.

Comparing the results of different systems of antigen-antibody reactions studied, it is apparent that gelatin sperm agglutination is most sensitive test to detect the antibodies against semen. This is in agreement with the findings of Scientists of Agricultural Research Station and Agricultural Experiment Station, Wisconsin.
(1962), who have shown the superiority of gelatin sperm agglutination test over the complement fixation test. Bratanov and Dikov (1960, 1962, 1965) have also advocated the use of sperm agglutination test to show the antibody titres in the sera of infertile cows.

It may be concluded from the results of the different systems of antigen-antibody reactions studied (Table 7) that the buck semen was undoubtedly antigenic, and the antibodies so produced were sperm agglutinins in the first place, had the power to agglutinate only such red cells that were adsorbed with soluble antigenic components of the whole semen and had a weak complement antigen-binding property.

It has been observed in the present investigation that bovine seminal plasma possesses haemolytic property which varies in concentration in different samples. This haemolytic property has also been observed by Millar (1956), Mitscherlich and Paufler (1960), Romanhink (1961) and Hunter and Hafs (1964). This haemolytic property of bovine seminal plasma was a major hinderence in conducting the indirect haemagglutination and complement fixation tests.

The biggest hurdle with complement fixation and indirect haemagglutination test has been the non-availability of suitable antigens and on account of this fact less work has been done on these tests in relation to antiseminal antibodies.

Although application of gel-diffusion technique
with semen of different species of animals had been done by various workers in order to analyse various precipitinogenic antibodies, but study on this aspect with buck semen had not been done as yet. The object of the present study was to analyse the various antigenic fractions of semen responsible for production of specific antibodies. Different fractions of semen namely seminal plasma and supernatant of freeze-thawed whole semen, washed seminal spermatozoa and epididymal spermatozoa were used to study their antigens. Hyperimmune sera prepared against buck semen in goats were used in the present test.

From the results of agar-gel-diffusion test with immune serum, it appears that the whole semen constitutes a complex antigens of which 10 could be recognised. Nine of these were present in seminal plasma, 5 in the seminal spermatozoa and 4 in the epididymal spermatozoa.

It was found from the results of gel diffusion test with immune sera absorbed by seminal plasma that 2 antigens were sper$^m$ specific and other antigens of semen were added as the sperms travelled through the accessory male genital tracts.

Buck semen has 10 antigens of which 9 are in seminal plasma and 4 in spermatozoa as compared to 17 and 16 antigens in buffalo semen and seminal plasma (Rao and Sadr, 1960), 7 and 5 in bull spermatozoa and seminal plasma (Hunter and Hafs, 1964; Matousek, 1964 c), 12 and 10 in ram semen and seminal plasma (Agar et al., 1965), 4 and 6 in
Ram acrosome and seminal plasma (Hathaway and Hartree, 1963), 15 and 16 in human semen and seminal plasma (Rao and Sadri, 1959), 3 and 6 in rabbit spermatozoa and seminal plasma (Rao and Bangalore, 1964) and 11 and 7 in guinea pig seminal plasma and sperm tail extracts (Pernot, 1956).

As it is apparent from the Table 8 & 9 that buck seminal plasma has 9 antigens and spermatozoa have 2 which are not common to seminal plasma, but the whole semen, instead of showing 11 antigens has shown only 10. The reason for this may be due to superimposition of some antigen-antibody precipitin lines.

Weil et al (1956) working with human semen have reported that seminal plasma and spermatozoa have the same number of antigens where as in the present finding it has been found that seminal spermatozoa have 5 antigens while seminal plasma has 9. What Weil et al (1956) have found with human semen, does not appear to be true with the buck semen.

Washed and freeze-thawed ejaculated spermatozoa have shown the presence of 5 antigens of which 2 are not present in the seminal plasma. Therefore, 3 antigens are common to both spermatozoa and seminal plasma. This may be due to permeability of the sperm cell wall. There are 2 possible ways to explain this observation: either the sperm liberates antigenic material into the seminal plasma or antigen is taken up by the sperm from the seminal plasma. It has been shown by Hetcher (1947) and Mann (1949) that
spermatzoza easily release hyaluronidase into the surrounding medium and hyaluronidase has been considered as one of the antigenic fraction of spermatzoza by Katsh (1959, 1961). Other reasons for release of spermatzoza antigens into the seminal plasma may be either the mechanical damage during centrifugation or the presence of metabolic products of the spermatzoza. The second alternative that spermatzoza may pick up the antigenic materials from the surrounding fluid as they travel through the accessory genital tracts, seems to be more correct. This finding also agrees with the work of Weil et al (1956), Weil (1960) and Weil and Rodenburg (1960).

Several workers (Landsteiner and Levine, 1926; Docto et al, 1952; Pernot, 1956; Gulbring, 1957; Hulpem and Weiner, 1961; Shahani and Sautham, 1962 and Edwards et al, 1964) have shown the antigenic relationship of spermatzoza with blood or serum. However, in the present study the presence of serum components in the development of precipitation lines was not excluded.

Since the vagina is a good absorptive surface, it is not inconceivable that antibodies against semen may be formed through vaginal absorption. But it is surprising that in the present investigation the transvaginal immunization did not induce either local or circulating antibodies in fresh goats. However, this finding is fully in agreement with the work of Isozima et al (1964) who failed to find detectable antibodies in the vaginal secretion or blood
serum of guinea pigs immunized solely by transvaginal methods.

Of particular interest was the observation that in goats previously hyperimmunized by the parenteral route, intravaginal instillation of the semen resulted in boosting up the circulating antibody titres. Even if the antibody titre had gone down it was still possible to evoke an anamnestic response as a result of subsequent instillation of the antigens into the vagina. This fact suggests that once the animals develop antibodies against semen, the possibility exists that fertility may be inhibited for a considerable period as a result of evoking an anamnestic reaction following subsequent mating or insemination.

The present anamnestic response in goats is well comparable with the findings of Behrman and Nakayama (1965) who got similar response in antibody titre on instillation of semen into the vagina or on mating of previously immunized female guinea pigs.

It is remarkable that the circulating antibodies can get excreted into the vagina as evidenced in the parenteral immunization, and the intravaginal application of antigen can boost up the circulating antibodies but can not induce the production of local antibodies. This may be due to the absence of such tissue or cells in the vagina of goat which would, presumably, respond to the application of antigen. If this be true, goat husbandry is relieved of at least one cause of female infertility, since
there appears to be no reason for parenterally introducing whole semen into a female goat. But the failure of hyper-immunized goats to conceive when mated with bucks, indicates that the antibodies in the genital tract, however low their titre may be, affected the fertility.

It is most interesting to note that goats immunized by parenteral route were not able to conceive even after repeated inseminations or matings. The experimental goats along with two controls were observed for a period of three months. Out of two control goats only one was found to be pregnant. Thus the percentage of fertility in control group can be taken as 50 while as in the immunized group the percentage was nil.

Taking into the account of results of previous workers as reviewed it is controversial whether a female animal can be rendered sterile by immunization with homologous sperm. However, several workers have reported lowered fertility in female rabbits (Dittler, 1920; Gyer, 1922; Jarcho, 1926; Behrman and Nakayama, 1925) in female guinea pigs (Castaro, 1926) and in female rats (McCartney, 1923; Fogelson, 1926) immunized with homologous sperm. No such observations regarding the effects of sperm immunization on the fertility of female goats have been made previously. In the present investigation it has been shown that immunization of female goats with homologous semen can lead to infertility for a period of at least three months. Even though the present investigation has been
based on less number of goats due to certain limitations, its various aspects of results for the practical application will prove of immense value in time to come.

The previous experiments gave an useful tool "sperm agglutination test" to investigate into the cases of unexplained infertility. The utility and the reactivity of this tool was examined by testing the sera collected from 140 cows and 15 heifers distributed into four different groups.

It was interesting to note that 80% of the infertile cows in which there was no demonstrable organic causes for failure of their conception, had circulating sperm agglutinins in titres ranging from 1:40 to 1:320. Contrary to this, the healthy and fertile cows and heifers as well, had no such agglutinin in titre above 1:20 which was considered as non-specific. It is remarkable that only 14% of the infertile cows with definite abnormalities, had sperm agglutinins in their sera.

The results obtained in the present study has a close similarity with the work of Bratanov and Dikov (1962) who conducted sperm agglutination test with the sera of 42 fertile cows in which titres did not exceed 1:16 where as serum-titres of repeat breeding cows went up to 1:512.

It was observed in the present study that there was variation in serum titre of a cow with spermatozoa from
bull to bull. This was noted specially with the sera of infertile cows allotted to the progeny testing programme at Government Cattle Farm, Patna. However, with very less number of such observations it was very difficult to reach at any definite conclusion regarding this variation and the average titre was recorded in the present study.

From the results of the present study it appears that there exists a definite relationship between the circulating antispermatozoal antibodies and unexplained infertility in cows. To explain the immunological infertility the following requirements must be met - the antibodies against semen should be formed and present in females; the antibodies would have to be present in the genital tract to react with their specific antigens.

Antigenic properties have been observed in bull spermatozoa (Docton et al, 1952; Gordon and Hunter, 1961; Matousek, 1964; Mittal et al, 1965) in the spermatozoa of buffalo (Rao and Sadri, 1960), and ram (Agar et al, 1965). It has also been shown that bovine seminal plasma is composed of proteins that are highly antigenic also (Larson et al, 1954). When a cow is inseminated or mated a quantity of semen is deposited in the uterus. In unfavourable circumstances or in susceptible cows, particularly those having traumatic lesions in their genital tract, the semen and its constituents may be absorbed giving rise to antibody formation. The results of the present study strongly suggest that semen deposited in the uterus at the time of
insemination or mating can provide an antigenic stimulus resulting in the elaboration of circulating anti spermatzoal antibodies in susceptible cows. These antibodies may appear in the genital tract and react with the antigen (sperm) in subsequent insemination or mating there by causing cytolysis of sperm or the antibodies may induce precipitation, agglutination or immobilization of sperm. This antigen-antibody reaction may either prevent the sperm from meeting the ovum or even if union occurs, it may cause early embryonic death.

The question arises as to whether or not antibodies against the seminal antigens present in the circulation are excreted into the lumen of the reproductive organs of cows. Unfortunately no attempt was made to demonstrate antibodies in the reproductive organs of repeat breeding cows in the present investigation. But in the study with the hyperimmunized goats, it was found that such antibodies do pass into the vaginal secretion though in very low titres but sufficient to cause infertility. Data are also available supporting this conception that antispermatozoal antibodies are excreted into the female genital tract in rabbits and guinea pigs parenterally immunized with homologous semen (Pomeranke, 1928; Parsons and Hyde, 1940; Edward, 1960; Ashitaka et al, 1964). It has been observed by Gupta et al (1962) that cervical fluids from repeat breeding cows had poor spermatozoal preservation capacity than did the fluids from normal fertile cows. Further it has been supported by Peterson
fertile cows. Further it has been supported by Peterson (1965) that uterine secretions from repeat breeding cows had depressing effect upon motility and oxygen consumption of bull spermatozoa as compared to easy breeders. It seems probable that such depressing effects upon sperm metabolism was due to the presence of anti spermatozoal antibodies in the uterine secretions of repeat breeding cow which finds the supports of Mittal et al (1965) and Matousek (1954 c) who have noted similar depressing effects of antiseminal antibodies upon the bull sperm metabolisms.

If antibodies do not pass from the circulation into the lumen of reproductive organs then the other possibility may be the local production of antibodies. The tissues of the reproductive organs of cows are able to produce antibodies against Brucella and Trichomonas organisms which can be demonstrated in the vaginal mucus or uterine washing but do not seem to pass into the circulation (Kerr, 1966; Kerr and Robertson, 1953). The antibodies against sperm or embryo may be produced locally in the uterus, cervix or vagina, and, may remain there to react with their specific antigens. Contrary to this Bratanov and Dikov (1965) showed that insemination of cows before uterine involution or in cases of endometritis led to the formation of high titres of sperm agglutinin in the sera of those cows. This clearly indicates that un repaired or traumatised uterine mucosa can absorb enough seminal antigen giving rise to high sperm agglutinin in the circulation.
circulation.

It would be more logical that antigen antibody reactions are responsible for lower fertility in repeat breeding cows, if it were demonstrated that specific antibodies against sperm were able to reduce fertilization rate or embryo survival. The results of work with cows (Menge et al, 1962 a) indicate that antibodies produced in cows against bull semen are able to cause fertilization failure or embryonic death when bull semen is treated with the antibodies before being used for insemination. Similarly, fertilization was prevented in rabbits inseminated with semen treated with homologous antibodies (Kiddy et al, 1959 b; Menge et al, 1962 b).

It can be concluded from the above findings that semen deposited in the uterus during insemination or mating can give rise to antibodies formation in susceptible cows. These antiseminal antibodies will react with semen in subsequent insemination resulting into fertilization failure or early embryonic death. Hence, if an economic herd of cow has to be maintained, insemination practices have to be so carefully and scientifically managed that every cow should conceive on single insemination. It is, therefore, suggested that in absence of other apparent factors causing infertility, the sperm agglutination test should be carried out. If immunological etiology is established, the same test
should be repeated with sperm of different bulls in the herd, to locate one whose sperm is not agglutinated in specific titre with the serum of that particular infertile cow and thus can be favourably used for insemination. The other method would be to give a sexual rest to the repeat breeding cow for a period of about four to five months as also suggested by Bratanov and Dikov (1964) so that the serum agglutinin may go down to a level where the conception is not affected. But this method would be less economical.
SUMMARY
SUMMARY

This study embodies the results of investigation on anti-seminal antibodies and their relation to infertility in livestock.

To study the antigenicity of buck semen, 10 female goats were immunized by parenteral and transvaginal routes. It was observed that buck semen was definitely antigenic as it induced the production of antibodies in female goats which could be assed by sperm agglutination, indirect haemagglutination, complement fixation and precipitation tests.

The sperm agglutination and sperm agglutination absorption tests with buck and bull spermatozoa revealed that hyperimmune sera prepared against buck semen were capable of agglutinating sperms of both species. This showed that buck semen had some antigenic fraction common with those of bull.

The agar-gel-diffusion test revealed at least 10 antigens in buck semen, 9 in seminal plasma, 5 in spermatozoa, of which 2 were sperm specific.

Transvaginal immunization could not induce antibody formation in healthy goats, however, in previously immunized goats by parenteral route, it was found to boost up the circulating antibody titre.

With the lapse of time after hyperimmunization by parenteral route, a rising tendency of antibodies in the vaginal secretions indicated the passage of circulating
antibodies in the genital tract. The fertility of the female goats was affected by hyperimmunization with buck semen.

During the immunological studies on goats, the gelatin sperm agglutination test was found to be easy and reliable and hence the same test was employed to study the immunological infertility in cows. It was observed that 80% of the repeat breeding cows in which there was no demonstrable organic causes for failure of their conception and 14% of those infertile cows which were having definite abnormalities, had circulating anti spermatozoal antibodies in titres ranging from 1:40 to 1:320. On the other hand the normally fertile cows and heifers had not such sperm agglutinins in their sera, in titres above 1:20. The results of the study strongly suggested that semen deposited during mating or insemination can provide an antigenic stimulus for the production of circulating antispermatozoal antibodies in susceptible cows. There was significant relationship between existence of circulating antispermatozoal antibodies and unexplained infertility in repeat breeding cows.

The possibility of these antibodies appearing in the lumen of the female reproductive organs and reacting with the sperms in subsequent in semination thereby causing cytolysis, precipitation, agglutination or immobilization of sperms has been indicated. This antigen–antibody reaction might be responsible for failure of fertilization or early embryonic death in repeat breeding cows.
It has been suggested that in absence of other factors causing infertility, sperm agglutination test should be carried out. On the basis of this test temporary infertility in such cows can be prevented by selecting a suitable bull for insemination or by allowing a period of sexual rest to such cows, so that the antibody titre may go down to a level where the conception is not affected.
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