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

1966

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

1966

B.P.S.R. Singh,
G.B.V.C., P.G., F.R.V.C.S.(Sweden),
Professor of Gynaecology & Sterility.

Post-Graduate Department
of
Gynaecology and Sterility,
Bihar Veterinary College,
PATNA.

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. Singh
1.XII.66

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

A C K N O W L E D G E M E N T S

C.V.Sc. and to Sri A.K. Prasad, M.V.Sc. for their well wishes, sympathy and encouragement received during the period of study.

I must place on record my thanks to the Animal Husbandry Department, Government of Bihar, for deputing me to undertake the higher studies.

(S. P. Pandey)

CONTENTS

"*****"
*

INTRODUCTION

I N T R O D U C T I O N

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 rela-
tion 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 steri-
lity are not available, it can well be imagined by an
estimate of Asdell (1955) 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, Tricho-
moniasis 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 fetilization 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 Metchi-
nikoff (1899) that the mammalian semen and its consti-
tuents, 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 steri-
lity. The subsequent available informations (Docton et al,
1952; Brabanov & Dikov, 1959; Menge et al, 1962) 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 Metchinikoff (1899) who tested for agglutinins and sperm immobilizing antibodies.

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

Farnum (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.

Hektone 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 precipiting indicating that the

seminal fluids were antigenic.

McCartney (1923) injected female rats sub-
cutaneously 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 comple-
tely the immune antibodies (from rabbits) to the A and
B antigens of human erythrocytes.

Pomerenke (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 mamma-
lian spermatozoa possessed both species and tissue xe
specificity. He proved that antibodies prepared in rabbits
against spermatozoa of bull, ram, guinea pig, rat and man
were species specific. Cross reactions occured 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 spermotozoa 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 anti-
genic 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, where-
as antispermatozoal sera did cross react with seminal
plasma.

Docton et al (1952) demonstrated the anti-
genicity 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 agglu-
tination 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-antigencity 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 pro-
vided 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 antiseminal 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 x 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 occured 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 immunoflurescent 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 erythrocyts 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 non of them appeared after absorption of the antiserm 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 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 (1964 a) 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 (1964 b) 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 homolo-
gous spermatozoa suppressed the reaction against homolo-
gous and heterologous germ cells. However, heterologous
spermatozoa could absorb agglutinins only against sper-
matozoa 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 demons-
trated the antigenicity of bull spermatozoa by some bio-
chemical 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 aginst
seminal and epididymal spermatozoa.

Rao and Bangalore (1964) conducted immunolo-
gical 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 spe-
cificity between seminal plasma proteins of bull, ram,
rabbit and Boar by agar-gel-electrophoresis.

Cvellar (1965) studied the antigenic proper-
ties 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 anti-
genic 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 sperma-
tozoa. 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 haemagglutina-
tion 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 inferti-
lity can be grouped under two headings (i) due to seminal
antibodies (ii) due to erythrocytic antibodies.

(i) Due to seminal antibodies.

The scientific beginning of this topic dates back to 1899-1900 when Landsteiner (1899) and Metchinikoff (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 varrying 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 andinhibition 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.

McCartney (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 hebavior 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.

Castaro (1926) induced temporary sterility for a period in guinea pigs by subscutaneous 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) discribed 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.

Pomerenke (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 seum 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 pretention 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 offsprings 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 muco-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 tire in their sera probably in response to the antigens which passed into the circu-laction, 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 correlationship between the amount of these agglu-tinins 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 occured to first service (42 cows) but when cows (total of 48) returned repeatedly to a given bull, titres upto 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 occured 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 inse-
minated with washed epididymal spermatozoa pretreated with
immune serum. They showed reduction in fertility rate in
immunized female guinea pigs, when artificially insemi-
nated. 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 concomitently
reduced from 28.6% 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 res-
ponses 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 insemi-
nation 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 instilla-
tion 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 infer-
tility, 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 immu-
nization 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 connec-
tion 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 varia-
tion 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 disappea-
rance 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 secre-
tions 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 occured when maternal antibodies were present against the foreign erythrocytic antigen. This condition occured 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 epitehlium. 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 calve'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 Metchinikoff (1899) independently discovered that it was possible to

induce the formation of antibodies against spermatozoa
in guinea pigs by means of parenteral injection of hetro-
logous sperm, several workers have explained male inferti-
lity to be caused by the presence of seminal antibodies.

Adler (1908) and Guyer (1922) demonstrated
that guinea pigs or rabbits can produce antibodies even
against their own spermatozoa, when injected with homolo-
gous 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 inhi-
bition 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 upto titre of 1:20 and 1:80 respectively. He
observed head, tail and mixed varieties of sperm aggluti-
nation. 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 vasdeferens 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 vasde-
ferens for the presence of sperm agglutinins. They detec-
ted 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
ta suspension. Other groups, of these species were sub-
jected to wounding of the testis or ligature of one or
both vasdeferens. 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 anti-genicity 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 macro-scopic 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 Trapany (1954) for detection of antispermatozoal
antibodies in rabbits. Rao and Sadri (1960) used the
highly sensitive haemagglutination technique of Boyden
(1951) and Stavitsky (1954), 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 anti-
body titre varrying 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 tra-nsvaginal 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 haema-
gglutination 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 anti-
body in 60 to 70% of female guinea pigs immunized against
homologous spermatozoa. Bull spermatozoa elicited anti-
bodies in 100 percent guinea pigs, the titre ranging between
1:25 to 1:410. They used spermatozoal suspensions containing
5×10^7 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 x

antigens of human seminal plasma. In their tests, rabbit immune sera, in serum dilutions varrying between 1:80 and 1:640, reacted in complement fixation test with seminal plasma in dilution upto 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 x 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 anti-typhoid serum. It has since been recognized that precipitating antibodies can be produced against most proteins and also against some polysacchrides. 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 varrying dilutions of antigen and then observing ring formation. They found that immune sera gave precipitation band with seminal plasma in dilutions upto 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 ante-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.

"********"

MATERIALS AND METHODS

MATERIALS AND METHODS

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:

Groups	No. of animals examined
Infertile cows without apparent anatomical and/or physiological abnormalities of genitalia.	75
Infertile cows with anatomical and/or physiological abnormalities of genitalia.	50
Fertile cows, pregnant or recently calved (control).	15
Heifers.(control).	15

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".

Rreparation 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. <u>Antigen for hyperimmunization:</u>

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. <u>Antigens for serological tests:</u>

(a) <u>Whole semen</u> :- Freshly collected semen was used for this purpose.

(b) <u>Seminal plasma</u> :- 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) <u>Freezed and thawed antigens:</u>- Freezing and thawing of semen or spermatozoal suspension in sline was carried out with the object of obtaining more of the water soluble spermatozoal antigens.

(i) <u>Whole Semen</u> : - Freshly collected and pooled semen.

(ii) <u>Washed seminal spermatozoa</u> :- 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

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 sline 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

in the deep freeze.

(iii) <u>Epididymal spermatozoa</u> :- 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 thermo-flask. 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 sperms/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 mertheolate 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 :-

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

S/C - Subcutaneous.

I/V - Intravenous.

Seven days after the last injection, each
animal was bled from jugular vein and the serum was sepe-
rated. 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) <u>By intravaginal route</u>:- Four goats, two new and two
from the above group were immunized by intravaginal route.
For this purpose a small sterile tampoon was soaked with
about two ml. of pooled buck whole semen containing only dead
sperm. The vulva was cleared with sterile saline and the
tampoon soaked in antigen was inserted into the anterior part
of vagina with the help of vaginal speculum. The tampoon 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 secretaions were obtained from each goat.

<u>Collection of vaginal secretion for detecting</u>
<u>the presence of antibodies</u> : - Vaginal secretion was collected
from both groups of immunized goats. For this a small sterile
cotton tampoon 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 conti-
nuously for elution of absorbed secretion and the tampoon was

pressed against the test tube wall and removed. Thus absorption and elution was performed for three times for each animal using the same tampoon and slaine. 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 contrl 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 throughly 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 occured in the lowest dilution of the inacti-vated 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.

<u>Preparation of phosphate buffer saline (PH 7.2):-</u> An M/15 solution of Primary Potassium Phosphate was prepared containing 9.078 gms. of Potassium dihrydogen Phosphate (KH_2PO_4) in a liter of Distilled water.

An M/15 solution of secondary Sodium Phosphate was prepared containing 23.896 gms. of Disolium Hydrogen Phosphate $(Na_2HPO_4, 12H_2O)$ in 1 litre of Distilled water.

Both the solutions were sterilized by auto-claving at 15 lbs. pressure for 30 minutes. 30 ml. of Primary Potassium Phosphate solution were added to 70 ml. of secon-dary Sodium Phosphate solution to give a buffer of pH7.2 and to it 0.85 gms. of Sodium Chloride was added.

<u>Adsorption of antigen on tannic acid treated erythrocytes:-</u> Equal volumes of 2.5% erythrocyte suspension and 1:10000 tannic acid in Phosphate buffer saline were mixed and incu-bated at $37^{\circ}C$. in a waterbath for ten minutes. It was centri-fuged, 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 concen-tration 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 be 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 accoring to the protocal given in Table 1.

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 erythro-cytes was obtained from Haffkein's Institute, Bombay. The titre of haemolysin used x was the highest dilution showing complete lysis of sheep R.B.C. The titration was done accor-ding to the method of Prasad (1966) as shown in the protocal 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.

<u>Preparation of sheep erythrocytes</u>: - The blood was collected
aseptically into an equal volume of modified Alsefver'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 referigerator 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.

<u>Preparation of indicator system</u>:- 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 con-
tained 2 M.H.D. of haemolysin.

<u>Preparation of serum</u> :- The serum was inactivated in a water-
bath at 56°C. for 30 minutes immediately before use.

<u>Anti complementary activity</u> :- Anti complementary activity
of the antigen and antiserm was found by the technique of
Kolmer <u>et al</u> (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.

<u>Complement fixation test</u>:- 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 comple-
te fixation of two units of complement.

T A B L E - I.

Protocol of the Indirect haemagglutination test.

Cavity No.	1	2	3	4	5	6	7	8	9	10
Phosphate buffer serum saline (pH 7.2) in ml.		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.25
Serum (1:5) in ml.	0.5	After mixing thoroughly 0.5 ml. of the contents from cavity No.1 was added to cavity No.2. The same process was repeated upto cavity No.8 from which 0.5 ml. of the contents was discarded.							0.25	
Dilution factor.	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	–	1:10
0.5% tanned and sensitised sheep erythrocytes in ml.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	–
0.5% tanned unsensitised sheep erythrocytes in ml.	–	–	–	–	–	–	–	–	–	0.5

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

The plate was kept overnight at room temperature.

T A B L E - II

Protocol for the titration of haemolysin

Tube No.	1	2	3	4	5	6	7	8	9	10
Normal saline (in ml.)	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.8	0.9	1.0
Haemolysin dilution (in ml.)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	–	0.1	–
	(1:100)	(1:200)	(1:400)	(1:800)	(1:1600)	(1:3200)	(1:6400)		(1:100)	
Guineapig complement. (1:20) (in ml.)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	–	–
2% sheep. erythrocyte suspension (in ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Control:-

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.

T A B L E - III

Protocol for the titration of the complement.

Tube No.	1	2	3	4	5	6	7
Normal saline (in ml.)	0.5	0.6	0.7	0.8	0.8	0.5	1.0
Guineapig complement (1:20)	0.5	0.4	0.3	0.2	0.1	0.5	-
2% sensitised sheep erythrocytes containing 4 M.H.D. of Haemolysin (in ml.)	0.5	0.5	0.5	0.5	0.5	.	0.5
2% unsensitised sheep erythrocytes.	-	-	-	-	-	0.5	-

Controls   Tube No.6 = Complement control.
Tube No.7 = Haemolysin control.

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

T A B L E - IV

Titration of anticomplementary unit of antigen or serum.

Tube No.	1	2	3	4	5	6	7	8	9
Antigen or serum undiluted (0.25 ml.)		1:2	1:4	1:8	1:16	1:32	1:64	-	-
Normal saline.	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.75
Complement 2 units (in ml.)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	-

The system was incubated at $37^{\circ}C$ in water bath for 30 minutes.

	1	2	3	4	5	6	7	8	9
2% sensitised cells containing 2 units of haemolysin (in ml.)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	-	0.25
2% unsensitized cells (in ml.)	-	-	-	-	-	-	-	0.25	-

The system was incubated in water bath at $37^{\circ}C$ for 30 minutes.

Controls   Tube No.8 = Haemolysin control
            Tube No. 9 = R.B.C. Control.

T A B L E - V.

Protocol of complement fixation test.

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12
Normal saline (in ml.)	0.40	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.80	0.50
Serum (in ml.)	0.10	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	0.25	-	-	-
Dilution factor.	1:5	1:10	1:20	1:40	1:80	1:160	1:320					
Antigen containing 4 antigenic units (in ml.)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	-	-	0.25	0.25	-
Complement 2 units (in ml.)	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.

2% sensitized cells containing 2 M.H.D. haemlysin (in ml.)	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. <u>Precipitation test</u> :

  (a) <u>Ring test</u> :-

        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 opalesent ring at the antigen-serum interphase read against a black back ground was noted as a positive reaction.

  (b) <u>Gel test</u> :-

        The technique employed for the preparation of agar gel was on the line of Ouchterlony (1948) with slight

modifications.

<u>Preparation of agar gel</u> :- "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 con-
centration 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.

<u>Precedure</u> :- 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 sorrounding cups with different
antigens. The charged plates and slides were kept under the

cover of Bell Jar at room temperature Anopen 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

R E S U L T S

Hyperimmunization :- In order to study the antigencity 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 one goat no.6.
Out of the two sera from healthy control goats only one, in
undiluted form, could agglutinate the sperm.

T A B L E No. 1

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

No. of animals	Immunized or control	Reciprocal of serum titre.												
		Undi-luted	5	10	20	40	80	160	320	640	1280	2560	5120	10240
G 1	Immunized.	+	+	+	+	+	+	+	+	+	+	+	±	-
G 2	"	+	+	+	+	+	+	+	+	+	+	+	±	-
G 3	"	+	+	+	+	+	+	+	+	+	+	±	±	-
G 4	"	+	+	+	+	+	+	+	+	+	+	±	-	-
G 5	"	+	+	+	+	+	+	+	+	+	+	+	-	-
G 6	"	+	+	+	+	+	+	+	+	+	+	±	-	-
G 7	Control.	+	-	-	-	-	-	-	-	-	-	-	-	-
G 8	"	+	±	-	-	-	-	-	-	-	-	-	-	-

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.

T A B L E No.2

Sperm agglutination test with antisera prepared against buck semen.

Animal number	Immunized or control	Reciprocal of serum titre with	
		Buck spermatozoa	Bull spermatozoa
G 1	Immunized.	2560	80
G 2	"	2560	160
G 3	"	2560	80
G 4	"	1280	40
G 5	"	2560	40
G 6	"	640	40
G 7	Control.	-	-
G 8	"	N	-

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.

T A B L E   NO.3

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

Semen absorbed with	Reciprocal of serum titre before absorption		Reciprocal of serum titre after absorption	
	Buck spermatozoa	Bull spermatozoa	Buck spermatozoa	Bull spermatozoa
Buck spermatozoa	2560	80	-	-
Bull spermatozoa	2560	80	320	-

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
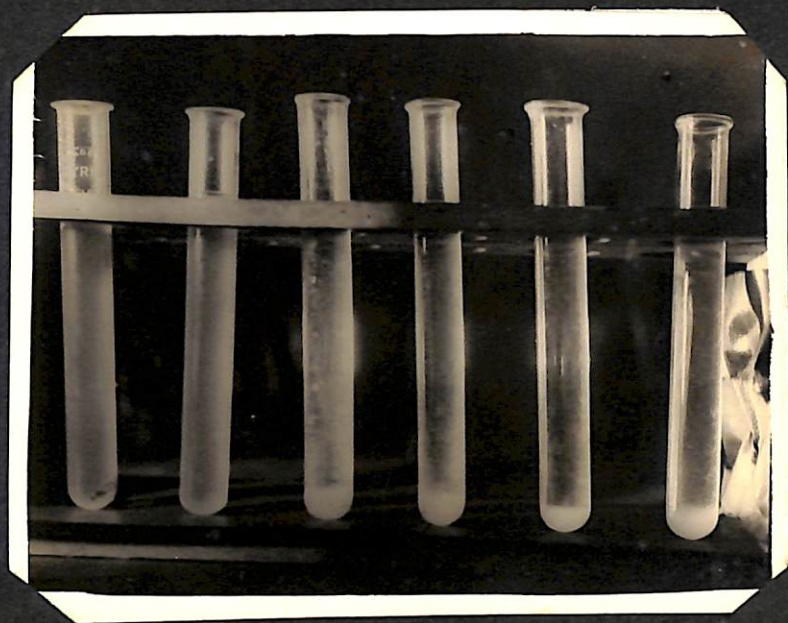
or head to tail. Within a few minutes, however, the
clumps of still actively motile spermatozoa became enta-
ngled with other clumps and with individual spermatozoa
(Plate-II). After 3-6 minutes the clumps were quite
large and continued to grow as long as some spermatozoa
remained motile. All the large clumps had a similar
appearance, which was predominantly that of "tail" agglu-
tination, 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.

T A B L E NO.4.

Antibodies in the vaginal secretion of the
immunized goats.

No. of goats	Reciprocal of sperm agglutination titre of vaginal secretions.			
	Before immunization	1 week after immunization	2 weeks after immunization	3 weeks after immunization
G 1	-	5	20	80
G 2	5	5	10	20
G 3	-	-	-	5
G 4	-	-	5	5
G 5	5	5	5	40
G 6	5	5	10	20

GRAPH NO. I

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
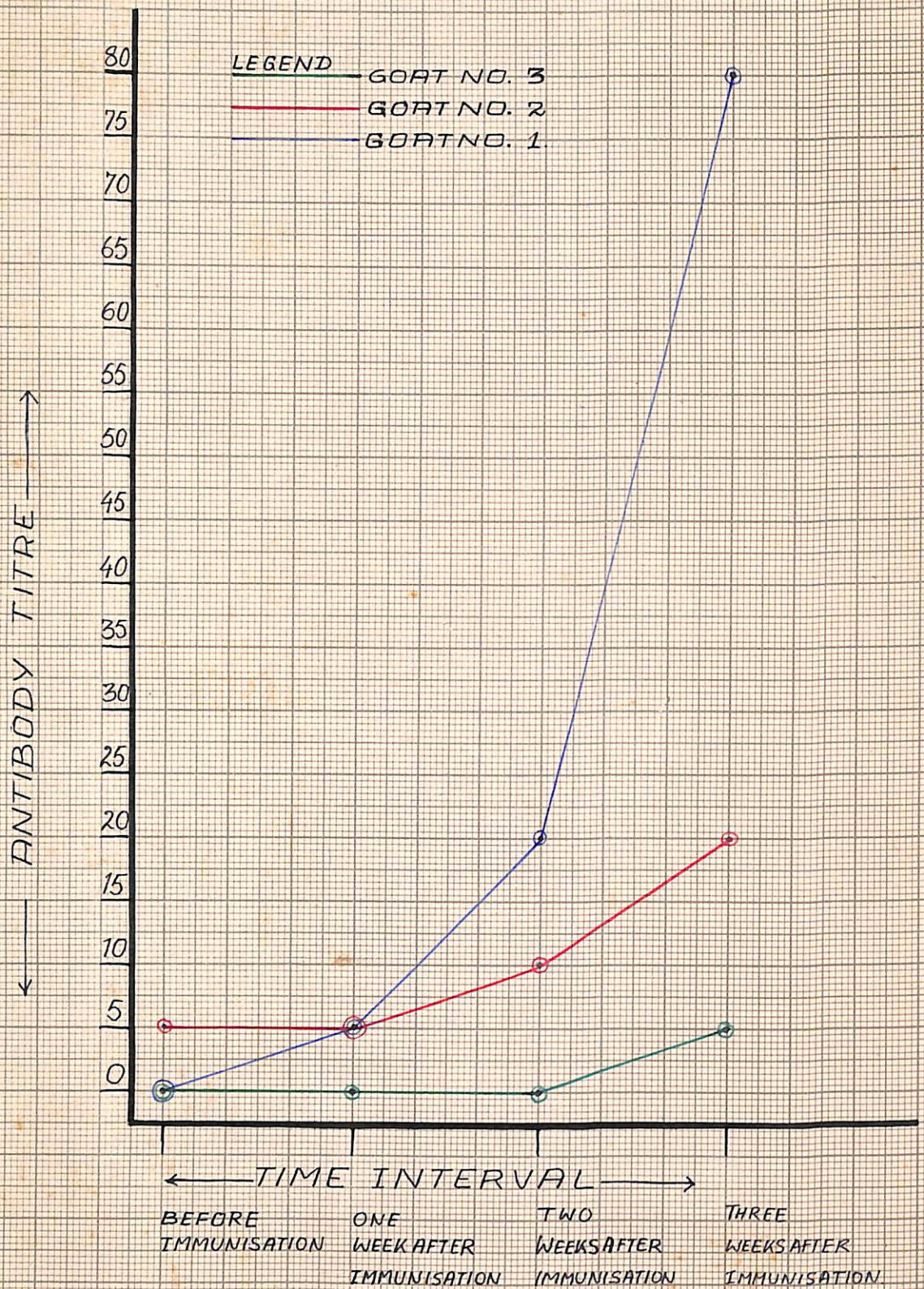
GRAPH NO. II

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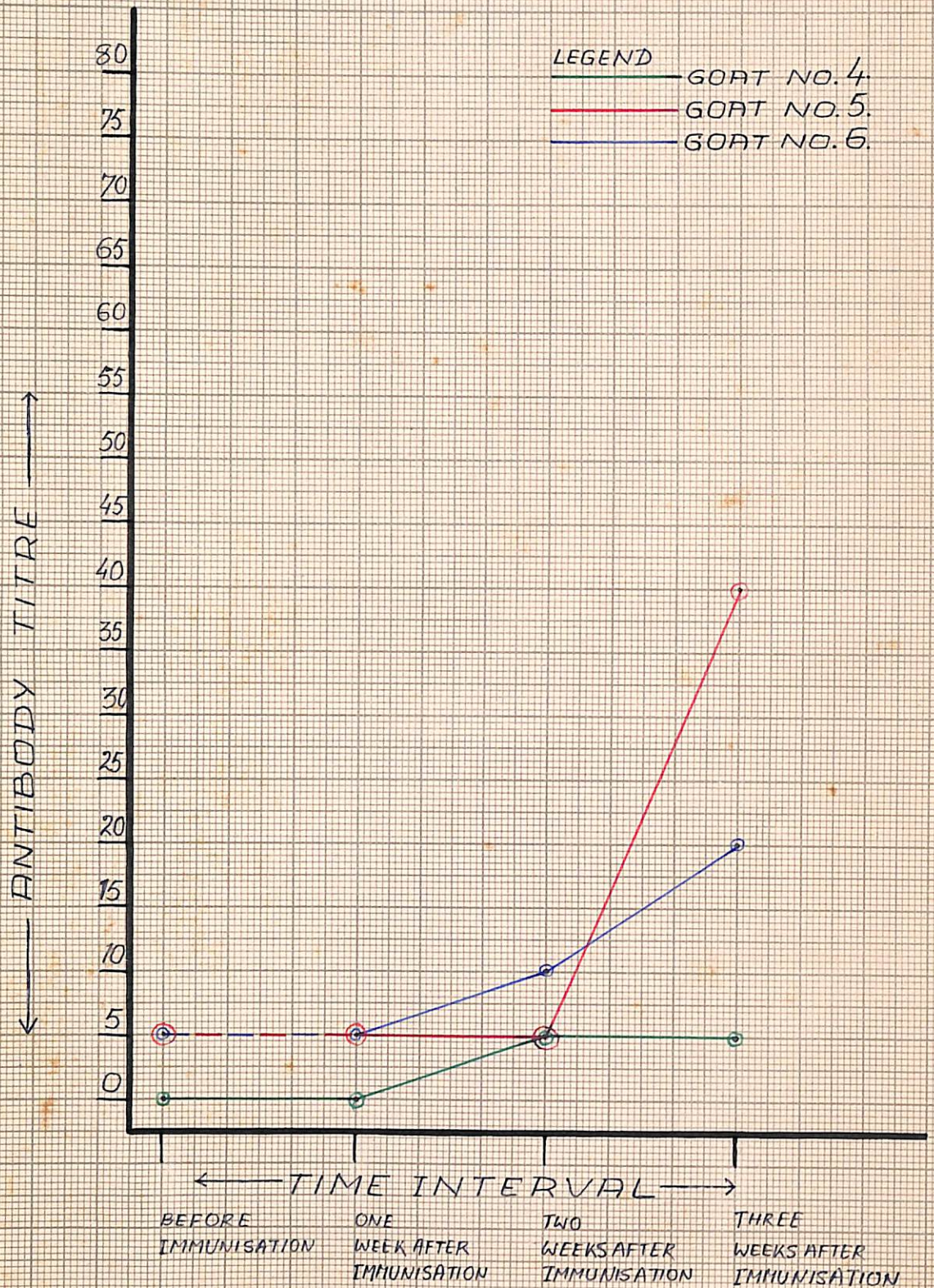
Significant rise in antibody titre in the vaginal secretion was observed in four out of six immunized goats three weeks after the hyperimmunization. However, rising tendency of the antibodies with the lapse of time after hyper immunization was not a regular feature.

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 upt 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.

COMPARISION 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 IN AGAR 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 anti-serum to whole buck semen was used, are presented in Table 8.

TABLE NO.5

Results of indirect haemagglutination test with the sera of goats immunized by parenteral route.

No. of animals.	Immunized or control.	Reciprocal of serum titre								
		10	20	40	80	160	320	640	1280	2560
G 1	Immunized.	+	+	+	+	+	+	+	±	-
G 2	"	+	+	+	+	+	+	+	±	-
G 3	"	+	+	+	+	+	+	+	±	-
G 4	"	+	+	+	+	+	+	±	±	-
G 5	"	+	+	+	+	+	+	+	±	-
G 6	"	+	+	+	+	+	+	+	±	-
G 7	Control	+	±	-	-	-	-	-	-	-
G 8	"	+	±	-	-	-	-	-	-	-

Note - + positive.
± Positive.
- Negative.

T A B L E No.6

Results of complement fixation test with sera of goats immunized by parenteral route.

No. of animals.	Immunized or control.	Reciprocal of serum titre.							
		5	10	20	40	80	160	320	640
G 1	Immunized.	+	+	+	+	+	+	±	-
G 2	"	+	+	+	±	-	-	-	-
G 3	"	+	+	+	+	+	±	-	-
G 4	"	+	+	+	+	+	±	-	-
G 5	"	+	+	+	±	-	-	-	-
G 6	"	+	+	+	±	-	-	-	-
G 7	Control.	-	-	-	-	-	-	-	-
G 8	"	-	-	-	-	-	-	-	-

Note :-   + No haemolysis.
± Incomplete haemolysis.
- Complete haemolysis.

TABLE NO.2

Results showing reciprocal of sperm agglutination, indirect haemagglutination
and complement fixation titre of hyper immune and control sera
of goats.

| No. of animals.	Immunized or control.	Reciprocal titre of sperm agglutination test.	Reciprocal titre of indirect haemagglutination test.	Reciprocal titre of complement fixation test.
G 1	Immunized.	2560	640	160
G 2	-do-	2560	640	20
G 3	-do-	2560	640	80
G 4	-do-	1280	320	160
G 5	-do-	2560	640	20
G 6	-do-	640	640	20
G 7	Control.	-	10	-
G 8	-do-	N	10	-

N - Agglutination in undiluted serum.
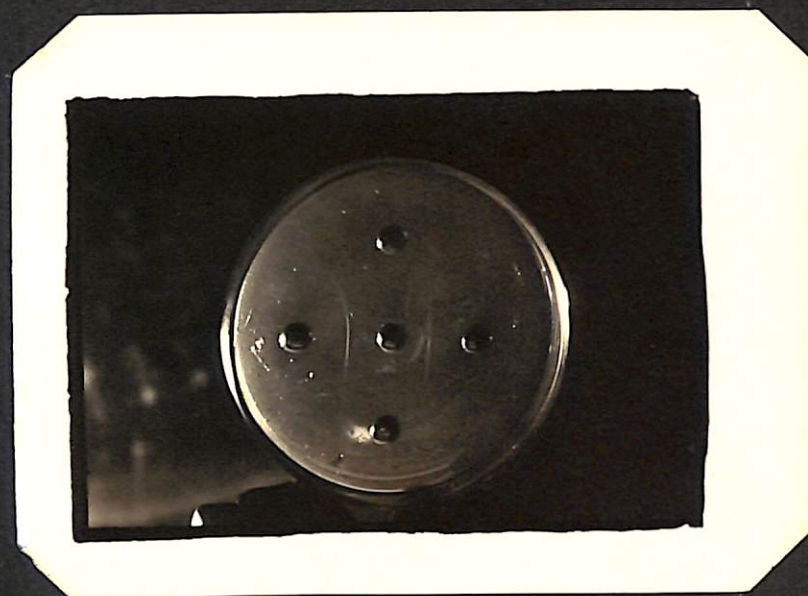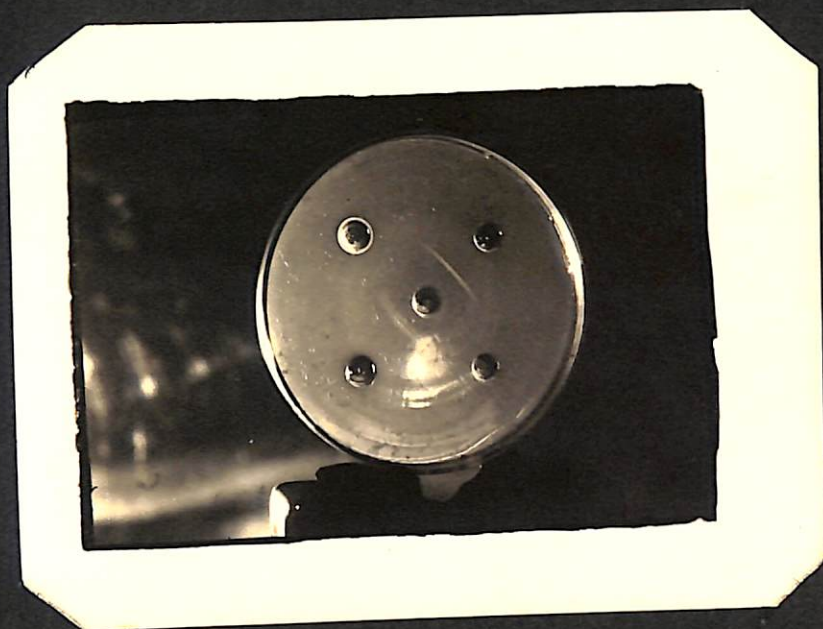
T A B L E NO. 8.

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

Antigens used	No. of precipitation lines formed		
	Moderate lines	Faint lines	Total
Freeze-thawed semen.	4	6	10
Seminal plasma.	2	7	9
Freeze-thawed washed spermatozoa.	-	5	5
Freeze-thawed epididymal spermatozoa.	-	4	4

The antisera prepared against whole buck
semen gave atleast 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 preci-
pitin 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 anti-
bodies 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.

Antigens used	No.of precipitin lines formed.		
	Moderate lines.	Faint lines	Total
Freeze-thawed semen.	1	1	2
Seminal plasma.	-	-	-
Freeze-thawed washed spermatozoa.	1	1	2
Freeze-thawed epididymal spermatozoa.	-	2	2

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.

T A B L E   NO. 10.

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

No. of goats	Reciprocal of titre after seven days.		Reciprocal of titre after 45 days.	
	Haemaggluti- nation.	Sperm agglu- tination.	Haemaggluti- nation.	Sperm aggluti- nation.
G 3	640	2560	40	160
G 4	320	1280	20	40

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 tampoon soaked in 2 ml. of buck whole semen was instilled into the vagina. Tampoon 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.

T A B L E NO. 11.

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

Goat no.	Immunized & control.	Reciprocal of titre before instillation of semen into vagina		Reciprocal of titre after instillation of semen into the vagina.	
		Haemagglu-tination.	Sperm aggln-tination.	Haemagglu-tination.	Sperm aggln-tination.
G 3	Immunised by parentral route.	40	160	160	640
G 4		20	40	160	160
G 9	Non-immunised fresh goats.	10	5	10	5
G 10		10	N	10	10
G 7	Control.	10	-	10	-
G 8	"	10	N	10	N

N = Agglutination in undiluted serum.

In goats number 3 and 4 which had been previously immunized by parenteral route, there was 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 No. 12

Results of sperm agglutination and indirect haemagglutination test with vaginal secretions of goats immunized by transvaginal method.

No. of goats.	Reciprocal of titre before instillation of semen into vagina.		Reciprocal of titre after instillation of semen into vagina.	
	Indirect haemagglutination	Sperm agglutination	Indirect haemagglutination	Sperm agglutination
G 3	10	10	10	8
G 8	—	—	—	—
G 4	—	10	8	5
G 9	—	5	5	5
G 10	—	5		

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.

T A B L E NO. 13.

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

	N	5	10	20	40	80	160	320	640
		R E C I P R O C A L O F T I T R E							
No. of reactors.	-	4	6	5	10	16	16	18	-
% of reactors.	-	5.3%	8%	6.6%	13.3%	21.3%	21.3%	24%	-
% of non-specific reactors.		20.0%					-		
% of specific reactors.		-					80.0%		

T A B L E NO. 14.

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

	N	5	10	20	40	80	160	320	640
		R E C I P R O C A L O F T I T R E S							
No. of reactors.	20	15	8	3	2	1	-	1	-
% of reactors.	40%	30%	16%	6%	4%	2%	-	2%	-
% of non-specific reactors.		92.0%					-		
% of specific reactors.		-					8.0%		

T A B L E NO. 15.

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

	RECIPROCAL OF TITRES								
	N	5	10	20	40	80	160	320	640
No. of reactors.	7	3	4	1	-	-	-	-	-
% of reactors.	46.6%	20%	26.6%	6.6%	-	-	-	-	-
% of non-specific reactors.		100%							
% of specific reactors.		-					Nil.		

T A B L E NO. 16.

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

	RECIPROCAL OF TITRES								
	N	5	10	20	40	80	160	320	640
No. of reactors.	10	3	2	-	-	-	-	-	-
% of reactors.	66.7%	20.0%	13.3%	-	-	-	-	-	-
% of non specific reactors.		100%							
% of specific reactors.		=					Nil.		

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.

T A B L E   NO. 17.

Results showing reciprocal of sperm agglutination
titre of sera of different groups
of cows and heifers.

Sera.	No. tested	Negative Titre (N to 20)	Positive		Percentage of positive.
			Weak Titre (40 to 80)	Strong Titre (160-320)	
Infertile cows without any apparent abnormalities.	75	15	26	34	80.0%
Infetile cows with apparent abnormalities.	50	46	31	1	8.0%
Fertile cows (control).	15	15	-	-	Nil.
Heifers (control).	15	15	-	-	Nil.

"*********"

DISCUSSION

DISCUSSION

In order to study the antigenicity of buck
semen six goats were hyperimmunized by parenteral route.
During hyperimmunization anaphylactic reaction was obser-
ved 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 invivo, but it indi-
cated 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.

Castaro (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 Castros' 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 (Docton 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 (McCartney, 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 (Pomerenke, 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 fractions 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 speices, 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 aggluti-
nation 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 discribed 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 (Pomerenke,
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 haemagglu-
tination 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 haemagglutina-
tion 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 demons-
trated 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 serolo-
gical 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), Romanink (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 precipi-
tinogenic 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 consti-
tutes 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 sperm-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

spermatozoa easily release hyaluronidase into the surroun-
ding medium and hyaluronidase has been considered as one
of the antigenic fraction of spermatozoa by Katsh (1959,
1961). Other reasons for release of spermatozoa antigens
into the seminal plasma may be either the machanical
damage during centrifugation or the presence of metabolic
products of the spermatozoa. The second alternative that
spermatozoa may pick up the antigenic materials from the
surrounding fluid as they travell through the accesory
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;
Docton et al, 1952; Pernot, 1956; Gulbring, 1957, Helpern
and Weiner, 1961; Shahani and Sautham, 1962 and Edwards
et al, 1964) have shown the antigenic relationship of
spermatozoa 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 immuni-
zation 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 atleast one cause of female infertility, since

there appears to be no reason for parentrally 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 alongwith 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 where 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 homolggous sperm. However, several workers have reported lowered fertility in feamle rabbits (Dittler, 1920; Gyer, 1922; Jarcho, 1926; Behrman and Nakayama, 1965) 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 atleast 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 applica-
tion 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
upto 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 un-explained 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 cir-cumstances or in susceptiable cows, particularly those having traumatic lesions in their genital tract, the semen and its constituents may be absorbed giving rise to anti-body 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 spermatozoal 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 occures, 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 anti-spermatozoal antibodies are excreted into the female genital tract in rabbits and guinea pigs parenterally immunized with homologous semen (Pomerenke, 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 metabo-
lism was due to the presence of anti spermatozoal anti-
bodies in the uterine secretions of repeat breeding cow
which finds the supports of Mittal at 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, 1965; 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 unrepaired
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 antibodoes 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 apparant 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 cows 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.

"*********"

S U M M A R Y

S U M M A R Y

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 assesed by sperm agglutination, indirect haemagglutination, complement fixation and pre-cipitation 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 anti-body formation in healthy goats, however, in previously immunized goats by parenteral route, it was found to boost up the circulating antibody titire.

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 susceptiable 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 either 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.

"*************"
***
*

REFERENCES

R E F E R E N C E S

1. Adler, H. (1908). Uber Autospermotoxin. Ztschr.
   Immunitatesforsch., 3 : 447.
   (cited by Rumke & Hellinga, 1959).

2. Agar, N.S., Rawat, J.S. & Roy, A.(1965). Antigens in
   ram semen and production of antibodies.
   Indian Vety. J., 42 : 727.

3. Asdell, S.A. (1955). Cattle fertility and sterility,
   little Brown and comp. Boston, Mass.
   (cited by Roberts, 1956).

4. Ashitaka, Y., Isojima, S. & Ukita, H. (1964 a).
   Mechanism of experimental sterility induced in
   guinea pigs by injection of homologous testis
   and sperm. I.Relationship between sterility
   and anaphylactic reaction of the uterus.
   Fertil & Steril., 15 : 206.

5. Ashitaka, Y., Isojima, S. & Ukita, H. (1964 b).
   Mechanism of experimental sterility induced in
   guinea pigs by injection of homologous testis
   and sperm. II. Relationship between sterility
   and sperm-immobilizing antibody. Fertil &
   Steril., 15 : 213.

6. Ardelt, F. (1931). Arch. Gynak.,145 : 474.
   (cited by Katsh, 1959).

7. Ayalon, N. (1964). "Sterilitas sine materia"-sterility
   not clinically diagnosable. 5th int. congr.
   Anim. Reprod. A.I. (Tranto), 5 : 47 (B).

8. Baskin, M.J. (1932). Temporary sterilization by the
   injection of human spermatozoa (a preliminary
   report). Amer. J. Obstet. Gynec., 24 : 892.

9. Baum, J. (1959). Reaction of guinea pig spermatozoa with
   homologous antibody. Lancet, 1 : 810.

10. Baum, J., Monger, J. L. and Schild, H. O. (1959).
    Immobilization of sperm by auto-antibodies.
    J. Physiol., 147 : 10.

11. Bechhold, H. (1905). Z. Physikal. Chem., 52 : 185.
    (cited by Kabat & Mayer, 1961).

12. Beck, J.S., Edward, R.G. and Young, M. R. (1962).Immune
    fluorescence technique and the isoantigenicity
    of mammalian spermatozoa. J. Reprod. Fertil.,
    4 : 103.

13. Behrman, S.J., Janusch, J.B., Hegler, R., Gershowitz, H. and Tew, W.L. (1960). ABO (H) blood incompatibility as a cause of infertility: a new concept. Amer. J. Obstet. Gynec., 79 : 847.

14. Behrman, S.J. and Nakayama, M. (1965). Antitestis antibodies; its inhibition of pregnancy. Fertil. & Steril., 16 : 37.

15. Behrman, S.J. and Otani, Y. (1963 a). Immunization of the guinea pig with homologous testis and sperm. Fertil. & Steril., 14 : 456.

16. Behrman, S.J. and Otani, Y. (1963 b). Transvaginal immunization of the guinea pig with homologous testis and epididymal sperm. Int. J. Fertil., 8 : 829.
(Anim. Breed. Abstr., 32 : No. 2302).

17. Behrman, S.J., Otani, Y., Porter, C.W. and Nakayama, M. (1963). Reduction of fertility in immunized guinea pigs. Int. J. Fertil., 8 : 835.
(Anim. Breed. Abstr., 32 : No. 2333).

18. Bennett, J.P. (1965). Quantitative comparisons of the proteins of the seminal plasma of bull, ram, rabbit and boar by agar-gel electrophoresis. J. Reprod. Fertil., 9 : 217.

19. Boyden, S.V. (1951). The absorption of proteins on erythrocytes treated with tannic acid and subsequent haemagglutination by antiprotein sera. J. exp. Med., 93 : 107.

20. Bratanov, K. and Dikov, V. (1959). Izsledvanija varhu spermoizoaglutinacijata vav vrazka sas zaplodjoe mostta na kravite. Nauc. Trud. naucnoizsled. Inst. Razud. Bol. izkustiv. Osemen, selskostop. Životn., 1 : 11.
(Anim. Breed. Abstr., 30 : No. 965).

21. Bratanov, C. and Dikov, V. (1961). Les reacherches sur le role des spermo-isoagglutinines dans la fecondation des vaches. Proc. 4th int. congr. Anim. Reprod., 3 : 463.

22. Bratanov, K. and Dikov, V. (1963). Studies on the effect of some immune bodies on the fertilization of farm animals. IV. Determination of antigenic characters of spermatoza, seminal plasma and erythrocytes in different species of farm animals. Izv. Inst. Biol. Patol. Razmnozhav. Selskostop. Zhivot., 4 : 15.
(Anim. Breed. Abstr., 34 : No. 22).

23. Bratanov, K., Dikov, V. and Papova, J.(1964). On the formation of auto-sperm antibodies in breeding animals. 5th int. congr. anim. Reprod. A.I.(Trento), 2:449.

24. Bratanov, K., Dikov, V., Radev, G. and Danov, D.(1965). Studies on the immunology of the reproduction. VI. The role of sperm antibodies in the insemination of cows with incomplete uterine involution and cows with latent endometritis. Vet. Med. Nauk., 2 : 323. (Anim. Breed. Abstr., 34 : No.213).

25. Brunner, E.K.(1941). Experiment in temporary immunization against pregnancy. Human Fertil., 6 : 10. (Cited by Katsh, 1959).

26. Castaro, R.(1926). Arch. di obstet. e Ginecol., 13 : 558. (Cited by Jarcho, 1926).

27. Chang, M.C.(1947). The effects of serum on spermatozoa. J. gen. Physiol., 30 : 321.

28. Cvellar, C.L.(1965). Antigenic properties of bovine and ovine semen, and effects of the antibodies on spermatozoa. Rev. Patron, Biol. anim. 9 : 23. (Vet. Bull., 36 : No.2413).

29. Dittler, R. (1920). Munchen med. Wchnschr,, 67 : 1495. (Cited by Kibrick et al, 1952).

30. Docton, F.L., Ferguson, L.C., Lazear, E.J.and Ely, F.(1952). The antigenicity of bovine spermatozoa. J. Dairy Sci. 35 : 706.

31. Edwards, R.G. (1960). Antigenicity of rabbit semen, bull semen and egg yolk after intravaginal or intramuscular injections into female rabbits. J. Reprod. Fetil., 1 : 385.

32. Edwards, R.G. (1964). Immunological control of fertility in female mice. Nature, 203 : 50.

33. Edwards, R.G. Ferguson, L.C. and Coombs, R.R.A.(1964). Blood groups antigens on Human spermatozoa. J. Reprod. Fertil., 7 : 153.

34. Farnum, C.G.(1901). The biologic test for semn. J.A.M.A., 37 : 172.

35. Fogelson, S.J.(1926). The non-specific antigenic effect of spermatozoa upon fertility. Surg. Gynec & obstet., 42 : 374.

36. Franklin, R.R. and Dukes, C.D. (1964). Antispermatozoal antibody and unexplained infertility. Amer. J. Obstet. Gynec., 89 : 6.

37. Freund, J., Lipton, M.M. and Thompson, G.E.(1953). A spermatogenesis in guinea pig induced by testicular tissue and adjuvants. J.exp.Med., 97 : 711.

38. Garcia Alfonso, C. and Perez Y Perez, F.(1959). Immunological infertility of a seminal character in female domestic animals. An.Inst.Invest.Vet. (Madr)., 9 : 285(B). (Vet.Bull., 30 : No.2020).

39. Gershowitz, H., Behrman, S.J. and Neel, J.V.(1958). Science,128 : 719. (Cited by solish et al,1961).

40. Gordon, M.J. and Hunter, A.G.(1961). Spermatozoa specific antigens in the bovine. J. Dairy Sci., 44 : 1182.

41. Gulbring, B.(1957). Investigation on the occurance of blood group antigens in spermatozoa from man and serological demonstration of the segregation of characters. Acta Med. Scand., 159 : 169. (Cited by Matousek, 1964).

42. Gupta, H.C., Branton, C., Evons, D.L. and Waters, W.H. (1962). Chemical and Physiological properties of the cervical and uterine fluids of repeat and normal breeding cows during oestrus. J. Dairy Sci., 45 : 668. (Abs).

43. Guyer, M.F. (1922). Studies on cytolysins. III. Experiments with spermatoxins. J.exp. Zool. 35 : 207.

44. Guyer, M.F. and Clause, P.E.(1933). Physiol. Zool., 6 : 253. (Cited by Katsh, 1959).

45. Hathaway, R.R. and Hartree, E.F.(1963). Observation on the mammalian acrosome: Experiemental removal of acrosomes from ram and bull spermatozoa. J. Reprod. Fertil., 5 : 225.

46. Hechter, O.(1947). Endocrinology, 41 : 204. (Cited by Rao and Sadri, 1960).

47. Hektone, L. and Manly, L.S.(1923). Specific preciptin reaction of semen. J. Infect. Dis., 32 : 167.

48. Helpern, K. and Wiener, A.S.(1961). Grouping of semen in cases of rape. Fertil. & steril., 12 : 551.

49. Henle, W.(1938). The specificity of some mammalian spermatozoa. J.Immunol., 34 : 325.

50. Henle, W., Henle, G. and Chambers, L.A.(1938), studies on the antigenic structure of some mammalian spermatozoa. J.exp. Med., 68 : 335.

51. Henle, W. and Henle, G. (1940). Spermatozoal antibodies and fertility. II. Attempts to induce temporary sterility in female guinea pigs by active immunization against spermatozoa. J. Immunol., 38 : 105.

52. Henle, W., Henle, G., Church, C.E. and Foster, I.(1940). Spermatozoal antibodies and fertility. I Attempts to induce temporary sterility in femal white mice by passive immunization with spermatozoal antisera. J.Immunol., 38 : 97.

53. Hunter, A.G. and Hafs, H.D.(1964). Antigenicity and cross reactions of bovine spermatozoa. J. Reprod. Fertil., 7.357.

54. Isozima, S. and Ashitaka, Y.(1964). Absorption of sperm antigen from the vagina in guinea pigs. Amer. J. obstet. Gynec., 88 : 433.

55. Isozima, S., Graham, R.M. and Graham, J.B.(1959). Sterility in female guinea pigs induced by injection with testis. Science, 129 : 44.

56. Jarcho, J. (1926). Artificial production of sterility with special reference to experimental temporary sterility biologically induced in the female. Amer. J.obstet. Gynec., 16 : 813.

57. Jones, F.S.(1927). J.exp. Med., 46 : 303. (Cited by Prasad, 1966).

58. Kabat, E.A.and Mayer, M.M.(1961). Experimental immunochemistry, 2nd edi, Charles, C. Thompson, Publishers, Spring-fields, Illinois, U.S.A.

59. Katsh, S.(1957). In vitro demonstration of utrine anaphylaxis in guinea pigs sensitized with homologous testis or sperm. Nature, 180 : 1047.

60. Katsh, S.(1958). Demonstration in vitro of anaphylactoid response of the uterus and eleum of guinea pigs injected with testis or sperm. j.exp.Med., 107 : 95.

61. Katsh, S.(1959). Anaphylactogenicity of hyaluronidase and an interspecies difference in the antigenicity of testicular hyaluronidase. Anat.Rec., 134 : 590 (Abs).

62. Katsh, S.(1959). Immunology, fertility and infer-
tility a historical survey. Amer. J.Obstet.
Gynec., 77 : 946.

63. Kerr, W.R.(1955). Vaginal and uterine antibodies in
cattle with particular reference to Br.
abortus. Brit.vet.,J., 111 : 169.

64. Krr, W.R. and Robertson, M.(1953). Active and passive
sensitization of the uterus of the cows in
vivo against trichomonas foetus antigen and
the evidence for the local production of anti-
body in that site. J.Hyg., 51 : 405.

65. Kibrick, S., Belding, D.L. and Merrill, B.(1952a).
Methods for the detection of antibodies
against mammalian spermatozoa. 1. A modi-
fied macroscopic agglutination test. Fertil.&
Steril., 3 : 419.

66. Kibrick, S., Belding. D.L. and Merrill.B.(1952 b).
Methods for the detection of antibodies
against mammalian spermatozoa. II.A gelatin
agglutination test. Fertil. & Steril., 3 : 430.

67. Kidder, H.E., Black, W.G., Wiltbank, J.N., Ulberg, L.C.
and Casida, L.E.(1954). Fertilization rates and embry-
onic death rates in cows bred to bulls of
different levels of fertility. J.Dairy
Sci., 37 : 691.

68. Kiddy, C.A., Stone, W.H. Tyler, W.J. and Casida, L.E.
(1958). Immunological studies on fertility and steri-
lity. I. An attempt to produce haemolytic
disease in cattle by isoimmunization. Proc.
4th Congr. Eur. Soc. Hemat. Acta. Hammatolo-
gica, 20 : 236.
(Vet. Bull., 59 : No.2840).

69. Kiddy, C.A., Stone, W.H., Tyler, W.J. and Casida, L.E.
(1959 a).Immunological studies on fertility and steri-
lity. III. Effect of isoimmunization with
blood and semen on fertility in cattle.
J. Dairy Sci., 42 : 100.

70. Kiddy, C.A., Stone, W.H. and Casida, L.E.(1959b). Immu-
nological studies on fertility and sterility.
II. Effects of Treatment of semen with antibodies
on fertility in rabbits. J.Immunol., 82 : 125.

71. Kolmer, J.A., Spoulding, E.H. and Robinson, H.W.(1951).
Approved Laboratory Technic 5th ed. Appleton-
Century-Crofts, New York.

72. Kostromin, N.E. and Kartashev, P.N.(1927). Jour.
        Microbiolojii, Pathologii, Infectzionich
        Bolezzy., 4 : 1.
        (Cited by Jarcho, 1926).

73. Kraus, R. (1897). Wiener Klin. Wochenschr., 10 : 736.
        (Cited by Kabat & Mayer (1961).

74. Laing, J.A. and Blackemore, F.(1951). Erythrocyte anti-
        bodies as a cause of abortion in cattle.
        Nature, 168 : 656.

75. Laing, J.A.(1955). Fertility and infertility in the
        domestic animals. 1st edi. Ballier, Tindall
        and Cox, London.

76. Landsteiner, K.(1899). Zur Kenntnis der spezifisch auf
        Blutkorperchen wirkenden Sera. Centralbl.
        Bakt., 25 : 546.
        (Cited by Katsh and Katsh, 1961).

77. Landsteiner, K. and Levine, P.(1926). On group specific
        substances in human spermatozoa. J.Immunol.,
        12 : 415.

78. Landy, M. and Trapani, R.J.(1954). A haemagglutination
        tests for plaque antibody with purified
        capsular antigen of Pasturella pestis.,
        Amer.J.Heyg.,59 : 150.

79. Larson, B.L., Gray, R.S. and Salisbury, G.W.(1954).
        The proteins of bovine seminal plasma.
        II. Ultra centrifugal and immunological
        studies and comparison with blood and milk
        serum. J. Biol. Chem., 211 : 43.

80. Lewis, J.H. (1941). The antigenic relationship of
        alcohal soluble substances of corpus luteum
        to those of testis and brain. Amer.J.Path.,
        17 : 725.

81. Mann, T.(1949) advances in enzymology, F.F.Ford,
        New YOrk.

82. Matousek, J.(1964 a). Antigenic characteristics of
        spermatozoa from bull, rams and boars.
        I. Erythrocytic antigens in bull spermatozoa.
        J. Reprod.Fertil., 8 : 1.

83. Matousek, J.(1964 b). Antigenic characteristics of
        spermatozoa from bull, rams and boars.
        II. sperm agglutinins in normal and immune
        sera. J. Reprod. Fertil., 8 : 5.

84. Matousek, J. (1964 c). Antigenic characteristics of spermatozoa from bull, rams and boars. III. Absorption analysis, precipitins and fructolysis in relation to the antigenicity of bull spermatozoa. J.Reprod. Fertil., 8 : 13.

85. Matsunaga, E. (1955). Amer. J. Human Gene., 7 : 66. (cited by Bahrman et al, 1960).

86. Mayer, A. (1922). Klin. Wchnschr., 1 : 1142. (cited by Jarcho, 1926).

87. McCartney, J.L. (1922). Studies on the mechanism of sterilisation of the female by spermatoxin. Amer. J. Physiol., 63 : 207.

88. McCartney, J.L. (1923). Further observations on the antigenic effects of semen. Mechanism of sterilization of female rat from injection of spermatozoa. Amer. J. Physiol., 66 : 404.

89. McLaren, A. (1964). Immunological control of fertility in female mice. Nature, 201 : 582.

90. McLaren, A. (1966). Studies on the immunization of mice with spermatozoa. Fertil. & Steril., 17 : 492.

91. Menge, A.C., Stone, W.H., Tyler, W.J. and Casida, L.E. (1962 a). Immunological studies on fertility and sterility. IV. Fertility of cattle and rabbits inseminated with semen treated with antibodies produced against semen, spermatozoa and erythrocytes. J.Reprod. Fertil., 3 : 331.

92. Menge, A.C., Kiddy, C.A., Stone, W.H. and Casida, L.E. (1962 b). Immunological studies on fertility and sterility. V. Effect of antibodies against semen and erythrocytes on rabbit embryos. J.Repord. Fertil., 4 : 87.

93. Metalnikoff, S. (1900). Etudes sur la spermatoxine. Ann. Inst. Pasteure, 14 : 577. (cited by Katsh & Katsh, 1961).

94. Metchinikoff, E. (1899). Etudis sur la resorption des cellules. Ann. Inst. Paseture, 13 : 937. (cited by Nakabayashi et al, 1961).

95. Millar, P.G. (1956). Observations on the presence of haemolysin in bovine semen. Brit. Vet.J., 112 : 106.

96. Mitscherlich, E. and Paufler, S. (1960). Uber eine
        hamolysierchde substanz im Bullensamen.
        Dtsch. tierarztl. Wschr.,67: 614.
        (cited by Hunter & Hafs, 1964).

97. Mittal, K.K., Salisbury, G. W., Graves, C. N. and
    Rasmusen, B.A. (1965). Antigens of bovine semen and
        the influence of specific rabbit anti-bull
        semen serum on metabolic activity of sperma-
        tozoa. J.Reprod. Fertil., 10 : 29.

98. Mudd, S. and Mudd, E. (1929). The specificity of
        mammalian spermatozoa with special reference
        to electrophoresis as a menas of serological
        differentiation. J. Immunol., 17 : 39.

99. Nakabayashi, N.T., Tyler, E.T. and Tyler, A. (1961).
        Immunological aspects of human infertility.
        Fertil. & Steril., 12 : 544.

100. Oslund, R.M. (1926). Physiological effects of spermato-
        xin. J.A.M.A., 86 : 1755.

101. Oudin, J. (1946). Compte. rend. Acad. Sc., 222 : 115.
        (cited by Mabat & Mayer, 1961).

102. Ouchterlony, O. (1948). Acta. Path. Microbiol. Scand.,
        25 : 186.

103. Parsons, E.I. and Hyde, R.R. (1940). An evaluation of
        spermato toxic sera in the prevention of
        preganancy. Amer. J. Hyg., B 31 : 89.

104. Pernot, E. (1956). Recherches sur les constituants anti-
        geniques des spermatozoides des cobayes. Bull.
        Soc. Chim. Biol., 38 : 1041.
        (cited by Hunter & Hafs, 1964).

105. Peterson, H.P. (1965). Some influences of uterine
        secretions from normal and repeat breeding cows
        on bovine spermatozoa. Amer.J.Vet.Res., 26:873.

106. Pfeiffer, H. (1901). Deutsche med. Wchnschr., 27 : 499.
        (cited by Katsch, 1959).

107. Phadke, A.M. and Padukone, K. (1964). Presence and
        significance of autoantibodies against sper-
        matozoa in the blood of men with obstructed
        vas diferens. J.Reprod. Fertil., 7 : 163.

108. Pommerenke, W.T. (1928). Effects of sperm injections
        into female rabbits. Physiol. Zool., 1 : 97.
        (cited by Katsh, 1959).

109. Prasad, A.K.(1966). Serological studies in Johne's
disease. M.V.Sc. Thesis, Agra University.

110. Rao, S.S. and Sadri, K.K. (1959). Proc. 6th International
Conference on Planned Parenthood. Ed. L.N.
Jackson.
(cited by Rao & Sadri, 1960).

111. Rao, S.S. and Sadri, K.K. (1960). The antigenic
composition of buffalo semen. J.comp.Path.,
70 : 1.

112. Rao, S.S. and Bangalore, S.A. (1963). Immunological
investigations with animal semen. Proc. 7th
Congr. int. planned parenth. Fed., Singapore.
(Anim. Breed. Abst., 33 : No. 2794).

113. Roberts, S.J. (1956). Veterinary obserics and genital
diseases. 1st ed. Ithaka, New York.

114. Romaniuk, J. (1961). The occurrence of haemolytic factor
in the seminal plasma of bulls. Med. met.,
17 : 395.
(Anim. breed. Abst., 32 : No. 1040).

115. Rumke, P. (1954). The presence of sperm antibodies in
the serum of 2 patients with oligozoozpermia.
Vox sang., 4 : 135.
(cited by Rumke and Hellinga, 1959).

116. Rumke, P. and Hellinga, G. (1959). Autoantibodies
against spermatozoa in sterile men.
Amer. J. Clin. Path., 32 : 357.

117. Savini, E. (1911). Immunite-spermotoxique et fecondation.
C.R.Soc. Biol., 71 : 22.
(cited by Katsh, 1959).

118. Shahani, S. and Southam, A.L. (1962). Immunofluoresent
study of the ABO blood antigens in human
spermatozoa. Amer.J. obstet. Gynec., 84 : 660.

119. Solish, G.I., Gershowitz, H. and Behrman S. J.(1961).
Occurrence and titre of isohaemagglutinins
in secretions of human uterine cervix. Proc.
Soc. exp. Biol. & Med., 108 : 645.

120. Smith, A.U. (1949 a). Some antigenic properties of
mammalian spermatozoa. Proc. roy.Soc., B 138:46.

121. Smith, A.U. (1949 b). The antigenic relationship of
some mammalian spermatozoa. Proc. roy. Soc.,
B 136 : 472.

122. Stavitsky. A.B. (1954). Micromethod for the study of
Proteins and antibodies. I. Procedure and
general application of haemagglutination and
haemagglutination inhibittion reactions with
tannic acid and protein treated red blood
cells. J. Immunol., 72 : 360.

123. Stevens, K.M., Fost, C.A. and Balows, A. (1965).
Circulating antibodies to human seminal plasma
in man. J. Reprod. Fertil., 10 : 137.

124. Struve, A. (1902). Deutsche med. Wchnschr., 28 : 425.
(cited by Katsh, 1959).

125. Tanabe, T.Y. and Casida, L.E. (1959). The nature of
reproductive failures in cows of low fertility.
J. Dairy Sci., 32 : 237.

126. Taylor, A.E. (1908). J. Biol. Chem., 5 : 311.
(cited by Katsh, 1959).

127. U.S.; Deprt. of Agri. (1962). Why infertility in
healthy cows ? Agric. Res.(U.S. Dep. Agric.).
11 : 13.

128. Von Moxter. D. (1900). Dentsche med. Wchnschr., 26 : 61.
(cited by Jarcho, 1926).

129. Vogt, E. (1922). Sterilität und Spermaimmunitat.
Klin. Wchnschr., 1 : 1144.
(Cited by Ashitaka et al, 1964 a).

130. Wang, Y. (1936). Effects of spermatozoan injections on
the fertility of female albino rats.
Chinease, J. Physiol, 10 : 53.
(cited by Ashitaka et al, 1964).

131. Weil, A.J. (1960). Immunological differentiation of
epididymal and seminal spermatozoa of rabbit.
Science, 131 : 1040.

132. Weil, A.J. (1961). Antigens of the adenexal glands of
the male genital tract. Fertil. & Steril.,
12 : 538.

133. Weil, A.U. and Roberts, C. (1965). Fertility of female
rabbits after iso-immunization with seminal
plasma. Fertil. & Steril., 16 : 356.

134. Weil, A.J., Kotsevalov, O. and Wilson, L. (1956).
Antigens of human seminal plasma. Proc.
Sec. exp. Biol. & Med., 92 : 606.

Weil, A.J. and Finkler, A. E. (1958). Antigens of
        rabbit semen. Proc.Soc.expr. Biol. &
        Med., 98 : 794.

Weil, A.J. and Finkler, A. E. (1959). Isoantigenicity
        of rabbit semen. Proc. Soc. exper. Biol. &
        Med., 102 : 624.

Weil, A.J. and Rodenburg, J. M. (1960). Immunological
        differentiation of human testicular
        (permatocele) and seminal spermatozoa.
        Proc.Soc. exper. Biol. & Med., 105 : 43.

Weil, A.J. and Roden burg, J.M. (1962). The seminal
        vesicle as the source of the spermatozoa-
        coating antigen of seminal plasma. Proc.Soc.
        exper. Biol. & Med., 109 : 567.

Wentworth, B. C. and Mellen, W. J. (1964). Effects
        of spermatozoal antibody and method of
        insemination on the fecundity of domestic
        hens. Brit. Poult. Sci., 5 : 59.

Wilson L. (1956). Sperm agglutination due to
        autoantibodies; A new cause of sterility.
        Fertil. & Steril., 7 : 262.

"************"
****
****
*****
****
**
$