DEVELOPMENT OF IMMUNOPROBES FOR SEXING SPERMATOZOA OF DAIRY CATTLE AND BUFFALO

Thesis

submitted to Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of

MASTER OF VETERINARY SCIENCE
IN
IMMUNOLOGY
(Minor Subject : Biotechnology)

By

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CATTLE
BUFFALOES

To

My husband

whose sacrifice and love made it possible

And to

All the support from Parents-in-law,

Parents

and the grace of God

CERTIFICATE I

This is to certify that the thesis entitled, **"DEVELOPMENT OF IMMUNOPROBES FOR SEXING SPERMATOZOA OF DAIRY CATTLE AND BUFFALO"** submitted to Punjab Agricultural University, Ludhiana, in partial fulfilment of the requirements for the degree of Master of Veterinary Sciences in the subject of Veterinary Immunology (Minor Subject : Biotechnology), is a bonafide research work carried out by Ripudaman Baweja (L-96-V-250-M) under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation has been fully acknowledged.

16/7/98

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CERTIFICATE - II

This is to certify that the thesis entitled, **"DEVELOPMENT OF IMMUNOPROBES FOR SEXING SPERMATOZOA OF DAIRY CATTLE AND BUFFALO"** submitted by **Ripudaman Baweja** (L-96-A-250-M) to the Punjab Agricultural University, Ludhiana, in partial fulfilment of the requirements for the degree of Master of Veterinary Science in the subject of Veterinary Immunology (Minor Subject : Biotechnology), has been approved by Student's Advisory Committee after an oral examination on the same in collaboration with an external examiner.

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ABSTRACT

In the present study, antisera to male specific antigen(s) was produced in female mice of C57BL/6 inbred strain using skin transplantation of skin grafts from the syngeneic male mice as primary immunization followed by first and third booster immunization with skin grafts and second and fourth booster immunization with intraperitoneal inoculations of spleen cell from the syngeneic male mice into the female mice. For immunization with skin graft transplantation, skin grafts from the tail of male mice were punched out (40 to 55 mm^2 size) using grafting punch, and grafted onto the reciprocal punched out skin sites of thorax region of female mice. The grafts of first skin transplantation experiments got rejected between 18 and 20 days post transplantation, second skin transplantation between 8 and 10 days and the third skin transplantation between 6 and 7 days post-transplantation, indicating immunization protocol was effective.

Sera from immunized female mice collected at different occasions of immunization were tested for antibody activity against cattle and buffalo spermatozoa. Indirect Immunofluorescence Assay (IFA) using goat anti-mouse polyvalent immunoglobulin FITC conjugate was conduted with an aim to develop immunoprobes for sexing spermatozoa of cattle and buffalo . Sera from early immunizations i.e. post primary immunization (Serum-I), first and second booster immunization (Serum-II and Serum-III respectively) did not have antibody activity as there was no fluorescence of spermatozoa with Serum-I or only 4-8 per cent with Serum-II and III, respectively. Serum post third and fourth booster immunization (Serum-IV and Serum-V respectively) were positive for antibody activity at 1:10 dilution and the

percentage of fluorescing spermatozoa was approximately 54% in buffalo spermatozoa and 52% in cattle spermatozoa. Serum-IV and Serum-V were also tested at four fold higher dilution (1:40 dilution) and there was no detectable antibody activity in the IFA. The results showed that antibody titres were low in the antisera and antibody activity was detectable in the IFA only after hyperimmunization. Pattern of fluorescence observed was that in general the whole spermatozoa, including tail, showed fluorescence. The fluorescence was more intense in the acrosomal cap suggesting differential distribution of HY antigen.

Results of the present study showed that detection of male specific protein (HY antigen) was approximately on 50 per cent of cattle and buffalo spermatozoa, which suggests that the fluorescence positive ones could be Y-chromosome bearing spermatozoa and the non-fluorescing ones could be X-chromosome bearing spermatozoa. However, the present study did not prove it and is an important question which needs to be resolved in future studies.

The present study results will serve as a pre-requisite to further develop monoclonal antibodies to HY antigen so that large amounts of anti-HY immunoprobes having high specificity become available for further use in sorting bovine X and Y chromosome-bearing spermatozoa and used in artificial insemination. Producing offsprings of pre-determined sex, will have an immense potential in the livestock industry.

Signature of Major Advisor 16-7-98

Signature of Student 16/7/98

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CHAPTER 1

INTRODUCTION

Producing the offsprings of predetermined sex has been the goal of domestic livestock producers and research workers with obvious interests that have immense potential in the livestock industry whereby the sex ratios could be altered to fulfil the needs of mankind for efficient livestock farm management. In India, cattle and buffalo are the major source of milk and draft animals. In case of buffalo, it is also used for meat. Selection for female calf birth is obviously required for milk production, while birth of male calves demands their disposal. Due to the religious ethics, slaughtering of male cow calves is not permitted which poses management problems to the dairy farmers. On the other hand, under the National Planning of creating high pedigree mother bull farms, birth of male calf is desired both in cows and buffaloes to achieve the production of bulls of high pedigree. Thus, sex pre-selection of female or male calf birth could be of great help in planning animal breeding in the country for improving management and development of dairy industry.

Sex pre-selection can be done: (i) before fertilization, i.e., spermatozoa sexing followed by artificial insemination (AI); (ii) after fertilization, i.e., embryo sexing followed by embryo transfer (ET).

Embryo Sexing: Embryo sexing methods are classified as invasive or non-invasive depending upon whether or not a biopsy of embryonic tissue is required (Betteridge, 1989). Obviously, non-invasive methods are considered optimal because they maintain

the integrity of the embryo (Betteridge, 1989) and so are less likely to impair the potential for successful embryo transfer and implantation.

Amongst various invasive methods for sexing embryos mentioned in the literature, one of the most common method is polymerase chain reaction (PCR) based on detection of Y chromosome DNA in a single blastomere taken from the dissected embryo (Schroder et al., 1990; Appa Rao et al., 1993).. Using embryo splitting, one cell is used for sexing and the rest of the cells are frozen (considering all the cells to be identical) and later used for breeding.

The other method is karyotyping in which cells are microscopically examined for identification of X and Y chromosomes (King, 1984; Picard et al., 1985).

Amongst the non-invasive methods are (1) monitoring the X-linked enzyme activity (like H.P.R.T, A.P.R.T etc.) prior to X-chromosome inactivation (Williams, 1986; Monk et al., 1988) or (2) immunoreaction of embryos with antibodies to sex-specific surface antigen, i.e., the H-Y antigen have been used and monitored by: (i) Cytotoxicity assay (Krco et al., 1976) and (ii) Immunofluorescence assay (Anderson, 1987).

Practical utility of embryo sexing is dependent on the availability of successful standardized embryo transfer techniques. The embryo transfer technique for animal breeding based on embryo sexing is more tedious and disadvantageous than using presexed sperms for artificial insemination. It needs more

expertise, and involves individual handling of the embryos for sexing which could be damaging and also after sex pre-determination, freezing of embryos is required for embryo transfer technology (ETT). In India, embryo transfer in cattle is yet being done in experimental laboratories and still not applicable at field level. In buffaloes which, in certain areas, constitute more than 50 per cent of the dairy population, the physiological conditions are not yet fully understood for embryo transfer technology (ETT). Therefore, use of sexed embryos does not seem promising quite for some time due to unavailability of standard embryo transfer technology (ETT) in the country.

Spermatozoa Sexing

It involves separation of X and Y chromosome bearing sperms, followed by the use of sorted X or Y chromosome bearing sperm populations for artificial insemination. Artificial insemination is an already established technology and has become a conventional tool of animal breeding at field level in our country.

Various methods for sexing of spermatozoa are:

(i) Sperm sorting based on differences in DNA content using Hoechst dye 33342 method (Johnson et al., 1989; 1994). The principle of this method is that the X chromosomes carry more DNA than the Y chromosomes, and this fluorescent dye binds to DNA helix in an amount proportional to the amount of DNA. Based on this differential fluorescence of X and Y chromosome bearing sperms, fluorescence activated cell sorter (FACS) can be used to

separate the X and Y chromosome bearing sperms.

The disadvantage is that the difference in DNA content between the X and the Y chromosome is very less, there could be some overlap of X and Y chromosome bearing sperms in the sorting procedure. Thus, the fluorescence activated cell sorter (FACS) has to be tuned up to sort out minor differences in the intensity of fluorescence between the X and the Y chromosome bearing sperms. An objective of exclusive fluorescent staining of one type of sperm can be accomplished by using specific immunoprobes for male sex-specific determinants expressed on the Y chromosome bearing spermatozoa surface. Eichwald & Silmser (1955) showed that the male skin grafts transplanted onto females of the same inbred strain were rejected while all other skin graft combinations were accepted. This incompatibility was attributed to the male-specific antigen, i.e. the HY antigen. This H-Y antigen is associated with the heterogametic sex and the molecule conferring HY antigenicity is phylogenetically conserved.

HY antigen occurs in two forms: (i) Soluble and (ii) Cell-membrane associated form on male tissues.

Production of antisera to HY antigen could be done in inbred females which differ from the syngeneic males with respect to only the male specific antigens, such as HY antigen, using either grafting/transplanting the skin isografts of male mice onto female mice (Billingham, 1961) or inoculating the spleen cells of syngeneic male mice intra-peritoneally into inbred female mice over an extended period (Wachtel, 1974).

Production of monoclonal antibodies can be done by fusion between the spleen cells of hyperimmunized females and myeloma cells for infinite supply of monospecific antibodies (Booman et al., 1989).

It was speculated that antibodies to this antigen may be used to detect male specific factors on the embryos and spermatozoa and may be useful in sex determination.

Immunoreaction between the anti H-Y antibody (polyclonal/ monoclonal) with the HY antigen on embryos or spermatozoa can be monitored by serological tests (i) immunofluorescence assay (Anderson et al., 1987; Wachtel et al., 1984); (ii) cytotoxicity assay (Krco et al., 1976; Wachtel et al., 1984).

1) **Cytotoxicity assay (Complement - dependant):** The basic principle behind this test is that the anti- HY antisera is incubated with cells carrying HY antigen and later complement is added. Cells carrying HY antigen are lysed. The lysed cells represent those carrying HY antigen, i.e. males and the unlysed ones female (Goldberg et al., 1971). The disadvantage of cytotoxicity assay is that this procedure being cytocidal to the Y sperms leaves only the X sperms viable or intact and thus separation of intact Y chromosome bearing spermatozoa is not feasible. Alternate methods which could separate X and Y sperms, leaving both viable, will be a more practical advancement.

2) **Immunofluorescence assay:** The anti-HY antibody is reacted with HY antigen carrying cells (spermatozoa/embryo), the conjugate to mouse antibody is added and the fluorescing cells

are considered to be positive for HY antigen (Wachtel, 1984). The fluorescence activated cell sorter (FACS) can be employed to sort the fluorescing and non-fluorescing cells.

The present study was planned with the following main objectives :

(1) To raise antisera to male-specific antigen(s) using inbred mice strains.

(2) To study its reactivity with cattle and buffalo spermatozoa.

(3) Developing immunofluorescent assay with this immunoprobe for spermatozoa.

Availability of such polyclonal antibody probes will be a pre-requisite for development of monoclonal immunoprobes and also useful to plan future studies of sorting X and Y chromosome bearing spermatozoa using fluorescence activated cell sorter (FACS) and use for artificial insemination to analyse the pre-determined birth rates in domestic animals.

CHAPTER 2

REVIEW OF LITERATURE

Definition of HY antigen - a selective marker for sexing germplasm

Interest in controlling the sex of mammalian offspring has continued because of the economic impact sex control might have on livestock production (Garner et al., 1983).

Through repeated brother-sister matings, one is able to develop genetically uniform strains of laboratory animals, and it has become a rule of transplantation biology that grafts of skin and other tissues are accepted when exchanged among the members of the same gender of such strains (Snell and Stimpfling, 1966).

Eichwald and Silmser (1955) observed that female mice rejected skin grafts from the syngeneic males of the same strain and all other sex combinations, usually were accepted. Since this mouse strain is highly inbred and essentially homozygous, failure of grafts of male tissue on females must have been due to a particular requirement of male tissue.

Subsequent studies by several workers reported a "second set" phenomenon with male-to-female isografts. The lives of subsequent male grafts being demonstrably curtailed on females that had previously rejected male grafts (Eichwald and Silmser, 1955; Eichwald et al., 1957; Krohn, 1958; Sachs and Heller, 1958; Billingham and Silver (1959).

This transplantation antigen was defined as histocompatibility antigen associated with the Y sex chromosome named HY antigen (Billingham and Silvers, 1960).

Biological properties and functions of HY antigen

In inbred mice strains, the genes of MHC modify the amount of HY antigen expressed on various tissues (Wachtel et al., 1973; Krallova and Demant, 1976). Mouse strains identical at the MHC (major histocompatibility complex), that is having the same H-2 genotype, show male graft rejection (Klein, 1975).

Ohno (1977a,b) postulated that MHC antigens conjoined with β_2 m serve as anchorage sites for HY antigen.

HY antigen is associated with antigens of the MHC (Beutler et al., 1978).

Wachtel et al. (1975b); Wachtel (1983b and 1984) proposed that HY antigen is the mammalian testicular-organizing factor responsible for differentiation of the indifferent gonad into testis.

Information involved in the HY generating system is located normally on the short arm of the Y-chromosome (Koo et al., 1981).

Wolf (1981) concluded that the Y-chromosome has an essential role in HY antigen production because of the strong association between the presence of both the Y-chromosome and the HY antigen.

HY antigen is controlled by a gene on the Y-chromosome, is expressed on all normal male cells of mammals except erythrocytes and pre meiotic germ cells (Muller, 1981 and Wachtel, 1983a).

Wachtel et al. (1975b) and Ohno (1977) hypothesized that HY antigen may be involved in the primary sex determination of the male.

HY has been proposed as the differentiation signal that causes the formation of the testis from the undifferentiated gonad (Simpson, 1983).

HY is a single molecular species responsible for triggering the indifferent gonad to differentiate into the testis (Goldberg, 1988).

HY is thought to be a male-determining substance in mammals because of its almost perfect correlation with maleness among a variety of mammalian species (Shapiro, 1981).

The presence of the Y-chromosome is related to male differentiation. HY hypothesis suggested that the presence of a male specific antigen called HY controlled by a gene(s) on the Y-chromosome was responsible for the differentiation of the primitive gonad into a testis (Cuevas et al., 1990).

Wiberg (1985) proposed that a Y-chromosome bearing spermatozoa must have the necessary structural gene(s) on an autosome as well as a regulatory gene on the Y-chromosome.

The primary development of a male rather than a female gonad in mammals is determined by the presence of a Y-chromosome. The other property unique to the Y-chromosome is the occurrence of a cell-surface antigen (designated HY) which distinguishes male from female (Goldberg, 1988).

Immunological response to HY antigen

According to Eichwald and Silmser (1955, 1958); Billingham et al (1965) and Silvers (1968), the response of female mice to male-specific HY antigen, called the Eichwald and Silmser effect,

has been best characterized in the C57BL/6 mice strain.

Although female mice of some strains regularly reject male skin isografts, females of other strains usually accept such grafts i.e. interstrain variation of male to female graft rejection pattern. (Gasser and Silvers, 1972).

Although the Y-antigen is usually regarded as a weak or minor transplantation antigen, in some strains, notably C57BL/6, the median survival time of male to female grafts may be as short as 16.5 days (Billingham and Hings, 1981).

In the past, the murine system has been used mostly for the study of HY antigen (Eichwald and Silmser, 1955 and Silvers and Wachtel, 1977) and the subsequent production of HY antibodies (Shalev et al., 1978; Simpson, 1983). The usual procedure has been to immunize female mice by intraperitoneal or intrapedal injections of syngeneic male spleen cells over an extended period.

Genetic factors are very important in determining responsiveness, however, their responsiveness can be modified by immunization procedures (Simpson, 1983). However other factors can also influence the immune response to HY antigen. Sena et al. (1976) demonstrated that grafts of ear skin and tail skin from male donors significantly outlive those of a trunk skin when transplanted to syngeneic female hosts, an observation subsequently extended to the HY antigen (Silvers et al., 1977). Possible explanations for this disparity in graft survival are (a) variation in mucopolysaccharide content of the skin in different regions of the body (Mathieson et al., 1975) and (b)

variation in the density of distribution of epidermal langerhans cells (Bergstressor et al., 1980).

Phylogenetic conservation of HY antigen

Wachtel et al. (1975a,b) and Shalev et al. (1980) provided evidence that the HY antigen, recognized serologically, appears to have been largely conserved throughout the evolution of vertebrates and perhaps more widely.

Wachtel et al. (1974) and Wachtel et al. (1975a,b) reported that antiserum against mouse HY antigen reacts with antigen on heterogametic cells of other mammals (including the human), and of birds and amphibians, even where the female is the heterogametic sex.

Wachtel et al. (1975a) reported exclusive presene of HY antigen in the mammalian male cells. In mammals, the findings that male tissue from every species studied reacted as HY positive led to the speculation that HY antigen was a male determining substance (Wachtel et al., 1975b).

Serologically defined HY antigen has been highly conserved during vertebrate evolution (Muller and Wolf, 1979; Pechan et al., 1979).

Murine HY antisera cross reacts with HY antigen in both vertebrates (Silvers and Wachtel, 1977; Wachtel, 1983b) and invertebrates (Wachtel et al., 1975a). Use of HY antisera to detect HY antigen of a different species would thus seem feasible.

The cross-reactivity of HY antigen between mouse, rat and human was demonstrated by the specific removal of mouse HY antibodies by male rat and human, but not by female rat and human WBC (Shalev et al., 1978).

Specific antisera from female mice recognizes male cells of other species, as HY is highly conserved during evolution and can be used to detect human soluble HY antigen (Hall and Wachtel, 1980).

Murine anti-HY- antisera were shown to cross-react with HY on cells from numerous species ranging from fish to man (Wachtel, 1983).

Relationship between HY antigen recognized by antibody (serological system) and cell mediated immune response

Uncertainity remains as to the nature of the male-specific protein and perhaps more than one sex-specific protein exists (Simpson et al., 1982; Silvers et al., 1983; Wachtel et al., 1984).

It was determined that male grafts were rejected by otherwise histocompatible females of the same inbred strain and HY specific cytolytic T-cells were produced by these grafted mice. Females grafted with male skin produced antibody defining a serologically detectable male antigen (which may or may not be the same as HY antigen) (Goldberg, 1988).

Silvers et al. (1982) reviewed differences in immunological reactivity of cell-mediated systems for the detection of HY (transplantation) antigen as well as serological detection of HY antigen on various tissue types. They suggested two male-specific

proteins, HY (transplantation) antigen and a serologically detectable male antigen.

Silvers et al. (1982) provided evidence suggesting that male-specific antigen(s) defined by graft rejection or by cell mediated lysis are not identical to serologically defined male specific antigen. The former would be called HY antigen and later S.D.M. (serologically defined male) antigen.

Wachtel et al. (1984) reported cross-reactivity between a monoclonal HY antibody and both tissue HY (transplantation) and soluble (SDM) HY antigens, suggesting that these antigens are either the same or have very similar properties.

HY antigen is defined as a male histoincompatibility antigen that causes rejection of male skin grafts by female recipients of the same inbred strain of rodents. Male specific or HY antigen(s) are detected by cytotoxic T cells and antibodies (Muller, 1997).

Chemical properties of HY antigen

The first attempt to isolate serological HY antigen was made by Nagai et al. (1979). The culture medium of Daudi human male Burkitt lymphoma cells which secrete HY antigen was used as a source of soluble HY. An extremely hydrophobic protein fraction was isolated from the secreted Daudi proteins. This fraction was composed of protein polymers derived from molecular weight 18,000 subunits. The molecular weight 18,000 polymers absorbed anti-HY antiserum and the molecular weight 18,000 subunits preferentially got bound to bovine ovaries which appear to be endowed with a gonad specific receptor for HY antigen (Muller et al., 1978a).

Hall and Wachtel (1980) gave estimates for the size of serological HY. Using mouse anti-HY antiserum, they could immunoprecipitate among polypeptides of various molecular weights, a protein of molecular weight 16,000 to 18,000 from Daudi cell secreted proteins.

The estimated molecular weight of the specific immunoprecipitate of Daudi-secreted HY antigen was 15,000 to 18,000 Daltons (Hall et al., 1981).

Considering that the molecular weight 18,000 subunit isolated by Nagai et al. (1979) had the best HY receptor binding activity, it was speculated that HY antigen physiologically occurs as a monomer of molecular weight 18,000 (Muller et al., 1988).

The exact nature of the serological HY antigen is unknown. Although molecules of 18 K Da have been detected with antisera against the serological HY antigen (Wachtel, 1983c), proteins of larger molecular weights have also been reported (Bradley and Heslop, 1985b; Goldberg and Reilly, 1987).

Bradley and Heslop (1985a) identified 3 polypeptides with estimated molecular weight of above 200,000, 50,000 and 20,000.

HY antigen is a glycoprotein, the serological determinant most likely being a carbohydrate chain with a galactose-terminal residue (Shapiro and Erickson, 1981).

HY polypeptide is hydrophobic and made of 160 or 250 amino acid residues to which no more than 5 glucosamine residues are attached (Ohno et al., 1981).

The serological determinant recognized by anti-HY antibody is a glycoconjugate containing terminal non-reducing and internal galactosyl residue (Shapiro and Goldberg, 1984).

Amice et al. (1992) demonstrated that the serologically detected, male predominant HY antigen was a surface glycoprotein.

Source and distribution of HY antigen

HY antigen(s) occur in four different states (1) As an integral part of the plasma membrane of almost all male cells with exceptions of diploid germ cells (Zenzes et al., 1978) and perhaps murine erythrocytes (Crichton, 1980). (2) Attached to the membrane of human erythrocytes (Muller et al., 1980), (3) Free in solution (Muller et al., 1978b, 1980; Wachtel et al., 1980), (4) bound by its gonad-specific receptor (Muller et al., 1978b, 1979; Nagai et al., 1979; Wachtel and Hali, 1979).

The justification for using Daudi cell secretions as the source of HY antigen was based on the assumption that the testis organizing factor would have a highly conserved structure, a postulate supported by conservation on the surfaces of many vertebrate cells of serologically active HY component(s) (Wachtel et al., 1975a).

Brunner et al. (1984) confirmed the observations of Zenzes et al. (1978) and Nagai et al. (1979) providing clear cut evidence for secretion of HY in cells of the testis, notably the sertoli cells and also in cultured Daudi cells.

In as much as secretion of HY in Daudi cells is correlated with the reduced presence of the molecule at the cell surface

(Beutler et al., 1978) favouring the hypothesis of Ohno (1977a), according to which HY is anchored to the membrane of extragonadal cells in physical association with $ß_2m$ and products of MHC.

The rationale for postulating secretion of HY antigen as an essential factor in testis formation has been weakened by the accumulating evidence that neither the serologically detectable HY antigen nor the HY transplantation antigen is consistently associated with phenotypic males (Silvers et al., 1982).

Sertoli cells are closely associated with the germ cells during spermatogenesis (Bellve and O'Brian, 1983).

Bradley and Heslop (1988) suggested that the HY antigen appearing on the surface of male meiotic cells was derived from sertoli cells. On the other hand, evidence that HY antigen could not be detected on spermatogonia of neonatal testis but became expressed on late spermatocytes and remained present on spermatids suggested that the HY genes are also expressed in the haploid germ cells (Koo et al., 1973; Koo et al., 1979).

The putative HY antigen is secreted by differentiated sertoli cells (Zenzes et al., 1978) and by an embryonic sertoli cell precursor (Ohno et al., 1979; Ohno, 1979).

Soluble HY antigen has been reported in the supernatant fluids of Daudi cell cultures (Nagai et al., 1979), testicular cell preparations (Muller et al., 1978a) and sertoli cell cultures (Zenzes et al., 1978).

Muller et al. (1981) provided evidence that HY is secreted or shed into the surrounding medium and that it can be taken up

by receptors that are present in gonadal cells but not extragonadal cells.

According to the findings of Muller et al. (1978a) and Zenzes et al. (1978), HY antigen is secreted by sertoli cells. HY antigen is found in the serum of male mammals and in the supernatant of cultured testicular cells (Muller et al., 1978b). Furthermore, epididymal fluid seems to be rich in HY antigen.

HY antigen is detectable on almost all male mammalian cells and is actively secreted by testicular sertoli cells (Zenzes et al., 1978; Muller et al., 1978a). HY antigen occurs in two different states, soluble and as an integral part of the plasma membrane. To this respect, it resembles immunoglobulins that occur as soluble molecules and sessile in the plasma membrane of B cells.

Sertoli-cell secretion of HY seems to be a pre requisite for HY function (Muller, 1980).

Gore-langton et al. (1983) presented the findings which do not support the contention that sertoli cells secrete a protein having the properties of serologically detectable HY antigen.

Evidence against haploid expression of HY antigen on Y-bearing spermatozoa is that sertoli cells but not germ cells synthesize HY antigen (Brunner et al., 1984; Zenzes et al., 1978) and that spermatids have the ability to bind HY antigen (Koo et al., 1979), suggesting that spermatozoa passively become HY positive regardless of sex-chromosomal composition.

Tests to detect HY (male specific) antigen

Several tests to detect male-specific antigen, commonly called HY antigen are: (1) Graft rejection assay: In an isogeneic system, female recipients of some strains of rodents reject male but not female skin grafts (Eichwald and Silmser, 1955; Review Billingham and Hings, 1981); (2) Cell-mediated lysis tests: Immune (sensitized) lymphocytes from female mammals that have been exposed to male cells lyse male target cells in-vitro under appropriate conditions (Simpson and Gordon, 1977; Simpson et al., 1981); (3) Serological assays: Antisera against the male specific antigen could be raised in female rodents by immunization with male cells (Koo, 1981).

Goldberg et al. (1971) demonstrated that tissue rejection was accompanied by antibody formation to the presumptive HY antigen.

The complement-mediated, cytotoxicity test monitored by dye-exclusion is a sensitive technique for detecting cell surface antigens on nucleated cells. Cytotoxicity tests have been used to identify several antigens on mouse spermatozoa including HY (Boyse et al., 1964, 1970).

Goldberg et al. (1971) reported antiserum to this male-specific antigen was cytotoxic to sperm and could be used to detect the presence of this antigen on all male murine tissues tested using cytotoxicity assay with murine epididymal spermatozoa as a source of HY antigen, antiserum from sensitized female mice, and rabbit complement. The reaction of HY antiserum

with HY antigen on the sperm surface in the presence of complement causes lysis of sperm cell. Modifications of the original assay included replacement of spermatozoa by epidermal cells (Scheid et al., 1972), fixation of cells and use of vital stain (Dooher et al., 1977) and quantification of A.T.P. use by spermatozoa as a measure of cytotoxicity (Piedrahita and Anderson, 1985).

The assay for cytotoxic HY antibody could be best carried out on sperm or on male epidermal cells because other cells are insensitive to lysis by HY antibody and complement (Goldberg et al., 1979).

Detection of HY antigen on spermatozoa - a marker for sexing spermatozoa

Koo et al. (1973) visually localized HY antigen on mouse sperm using antibodies against the HY antigen and tobacco mosaic virus (as visual marker). The pattern of labelling HY with tobacco mosaic virus implied the presence of HY in the acrosomal cap, little or no labelling in the post-acrosomal region, connecting piece, mid-piece and tail. The disproportionate representation of HY on the sperm surface led quite naturally to attempts to eliminate Y-bearing cells with HY antibodies and thereby to influence the sex-ratio among the progeny of females inseminated with serum-treated spermatozoa. The expectation was that a preponderance of HY in Y-bearing sperm could provide a basis for immunoselection.

Bradley et al. (1986) reported detection of HY (male-specific) antigen on the plasma membrane of both Caput and Caudal

ram spermatozoa. In these spermatozoa, the distribution of HY antigen was confined to both the posterior region of the head and the mid-piece region of the flagellum. In addition, Caput spermatozoa exhibited on the flagellum at the base of the head on the plasma membrane from flagella of caudal spermatozoa, a male specific protein with molecular weight 25-27 Kd.

Using indirect immunofluorescence technique, the HY antigen was localized on the acrosomal membrane of mouse epididymal and washed ejaculated human spermatozoa and on the entire membrane of male mouse splenocyte and thymocyte. Immunohistochemical localization of the antigen in the testicular secretion indicated HY antigen(s) presence on the membrane of sperm heads (Iyer et al., 1989).

There are lack of reproducible serological tests (HY antigen). Many of the difficulties in this field relate to the unsatisfactory nature of the available serological tests. The background noise in most tests is high and both polyclonal antisera and monoclonal antibodies have low titers. This has precluded the use of such antibodies making serological assays of HY extremely difficult (Koo, 1981).

Analysis of male-specific HY antigen is difficult, in part, because of the tendency of male specific antisera to bind cell surface components found in male and female cells (Brunner et al., 1988).

Booman et al. (1989) reported that, only a low percentage of mice show a good antibody response to HY antigen and their sera

are usually low titered and of low affinity.

The anti-HY antisera have been assayed using a variety of methods, most of which have required repeated testing to ensure the validity of the results (Koo, 1981).

Since conventionally produced polyclonal antibody in HY antisera has low titers and the methodology for HY detection differs among several investigators, it has been difficult to quantitate and compare experimental results (Goodfellow and Andrews, 1982).

Use of polyclonal antisera to HY antigen may give rise to relatively high degree of non-specific background (Piedrahita and Anderson, 1985).

It can be concluded from the above literature that HY tests are difficult to perform and require experience and adequate training of the investigator. Difficulties in HY testing arise from the following: (a) Their titer is comparatively low (ranging from 1:2 to 1:32); (b) The quality of target cells for detection of antiserum activity is essential. The preparation of viable cells in the procedure is critical. The cytotoxicity test using Raji cells as targets is susceptible to errors due to the dependency of HY expression on the growth phase of Raji cells, and non-specific cross-reactivity of the mouse/rat antisera with the human Raji cells (Muller, 1982; Goodfellow and Andrews, 1982). (c) Differential tissue distribution of HY antigen makes appropriate controls necessary. If carefully performed, however, HY tests are no less reliable than many immunological assays

(Muller, 1982).

Bradley and Heslop (1985a) introduced a method for the production of high titer HY antisera. Antisera with titers as high as 1:200 to 1:400 were obtained by transplanting syngeneic male skin into the spleen of female recipient. This was a great breakthrough of HY serology considering that conventional anti-HY antisera had titers in the range of 1:2 to 1:32 using each high titer antisera for the immunoprecipitation of Daudi secreted proteins.

Haploid expression of HY antigen

An immunologic approach to separation of sperms is based on haploid expression of X- or Y-linked genes provided that some of these gene products are present on the sperm plasma membrane. Because the expression of HY antigen requires genes on the X- as well as the Y-chromosome, a haploid Y-chromosome-bearing spermatozoa could not produce HY antigen which suggests that all spermatozoa may have acquired the antigen from sertoli cells (Ohno et al., 1982).

Specific HY antibody binds to approximately half of mammalian sperm as a result of haploid gene expression of Y-chromosome bearing spermatozoa (Ali et al., 1990).

Immunological markers for separation of X and Y chromosome bearing spermatozoa

There are at present two potential ways of influencing the sex ratio. (1) separation of sperm into viable fractions of X and Y bearing sperms (van Vliet et al., 1989) and (2) the diagnosis of

the sex of pre-implantation embryos (van Vliet et al., 1989).

Although numerous schemes have been devised and promoted for the putative separation of X and Y chromosome bearing spermatozoa, none has gained widespread acceptance. The possibility that Y-chromosome bearing spermatozoa express a male-specific antigen on the plasma membrane, but that the same antigen is absent from X-chromosome bearing sperm has been re-examined. Immuno-histochemical studies with high titer serologically detectable, male-specific antiserum revealed that serologically detectable, male specific antigen is present on approximately 50% of spermatozoa and is located on both the post-acrosomal region of the head and the mid piece of the flagellum. Similar results have also been described by other investigators working with bull spermatozoa (Bradley, 1989).

The percentage of sperm lysed specifically by anti-HY antibody often exceeded 50 per cent, suggesting that atleast some of the X-bearing sperm possess the Y antigen. This is important as it suggests that the Y-antigenic constitution of mouse sperm is determined by its precursor cells rather than the genetic material contained in the sperm. If borne-out, this would preclude the use of immunological methods of either distinguishing or separating X or Y bearing spermatozoa (Gasser and Silvers, 1973).

The proportion of epididymal spermatozoa that are HY positive is greater than 50% (Koo and Goldberg, 1978; Koo et al., 1973; Zaborski, 1979), while normally one would expect to find

equal proportions of X- and Y-bearing spermatozoa.

Bennett and Boyse (1973) artificially inseminated mice with spermatozoa that had been exposed in-vitro to HY antibody and complement and reported a slight if significant skewing of sex ratio in the offspring of female mice inseminated with sperm pooled from epididymis and vas deferens and reacted with conventional HY antiserum and complement; control litters contained 53 per cent males while there were 45% males in treated litters.

Bryant (1980) and Ali et al. (1990) investigated and reported that the sex ratio of murine offspring after treatment of spermatozoa with HY antiserum was significantly altered.

Of numerous methods to separate sperm into two sex-specific fractions, only flow cytometry as described by Garner et al. (1983) has been successful (Johnson, 1988). The preparation for flow cytometry can interfere with the viability of the sperms, but Johnson et al. (1989) described modifications which were compatible with sperm survival in rabbits.

Ali et al. (1990) summarized that several approaches have been applied to the problem of separating Y- from X-chromosome bearing spermatozoa. Among the separation methods are centrifugation, galvanization (based on net electric charge), the use of antibody probes and quantitation of DNA by fluorescent stains. Centrifugation and galvanization appear to be ineffective. Treatment with antibodies to sperm cell surface antigens has been unsuccessful possibly as a result of

experimental variables including antigen-antibody specificity, sorting technique, and consistency of application. Utilization of DNA fluorescent stains (Johnson et al., 1989) and their effects on sperm viability and fertilizing capability (Johnson, 1988 and Johnson et al., 1989) are under investigation.

Techniques for separation of X- and Y-bearing spermatozoa have not withstood testing in controlled experiments involving agriculturally important domestic animals (Amann and Seidel, 1983).

Ali et al. (1990) demonstrated that HY antigen appears to be expressed on the plasma membrane of haploid Y-chromosome bearing bovine spermatozoa, and monoclonal HY antibody can be used in conjunction with fluorescence activated cell sorter to enrich the proportion of Y-or X-chromosome bearing spermatozoa in semen. Before applying this technology for control of sex ratio in cattle, insemination of cows with samples containing sorted spermatozoa will provide a final test to evaluate fertility of the processed semen.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental animals

Inbred C57BL/6 male and female mice of 8-10 weeks age and weighing 18-22 grams were used. Mice were procured from National Institute of Immunology, Aruna Asaf Marg, New Delhi. These animals were maintained at Animal House, Department of Veterinary Immunology, Punjab Agricultural University, Ludhiana. Feed for mice was procured from M/s Jwanda Feeds, Village Shankar, Ludhiana. These mice were used for raising antisera against the H-Y antigen.

3.2 Production of antisera to male specific antigen(HY antigen)

HY antibodies were raised in adult C57BL/6 female mice using immunization with skin transplants and spleen cell inoculations from the syngeneic male mice. The details are given below:

Plan of the Immunization

3.2.1 Skin transplantation

Male mice were used as donor of skin grafts and female mice as recipient of skin grafts. The skin transplantation was done essentially according to the technique described by Billingham (1961).

3.2.1.1 Preparation of donor male mice for obtaining skin grafts

Male mice were anaesthetized with Ketamine (0.2 ml/20 g mice, intramuscularly) and maintained with anaesthetic ether and secured to a small operating board. Circular skin grafts of 40-55 mm^2 size were punched out from the tail skin with the help of a

grafting punch. From one animal, 2 or 3 grafts were taken.

3.2.1.2 **Preparation of recipient female mice for skin graft transplantation**

Female mice were anaesthetized with Ketamine (0.2 ml/20 g mouse, intramuscularly) and maintained with anaesthetic ether and secured to a small operating board. The thorax region was prepared for transplantation of grafts by shaving off the hair at the site. Circular pieces of skin grafts were punched out from the thorax region to create sites for transplantation of the male syngeneic skin grafts. Male mice tail skin grafts were fixed in place onto the female mice thorax region. The grafts were covered with a strip of adhesive bandage (Steri-strip Sterile) as dressings.

The dressings were removed 4-5 days after transplantation and graft survival appraised. The day, the graft got totally dislodged was scored as the day of rejection.

Primary and Secondary (Booster) Immunization

Skin graft transplantation was used for primary immunization. Secondary/booster immunizations were done using either skin transplantation and at some intervals using intra-peritoneal inoculation of female mice with spleen cells from the syngeneic male mice (Protocol shown in Table 1).

Preparation of skin grafts and transplantation was done similarly for both primary and secondary immunization. Skin transplantation was done with the help of Dr K.K. Mirakhur, Department of Veterinary Surgery & Radiology.

3.2.2.1 Preparation of spleen cells for secondary/booster immunization

Male mice were anaesthetized with anaesthetic ether. They were then sacrificed by the cervical dislocation of spinal cord. These were immersed in 70% alcohol to disinfect externally. Spleens were dissected out and teased with forceps on stainless steel wire mesh kept in a petriplate containing serum free RPMI-1640 to remove connective tissue and homogenized for dispersion of cells. Then, the solution containing dispersed spleen cells in RPMI-1640 was centrifuged using Sorvall Centrifuge at 1200 rpm for 10 minutes. Supernatant was discarded and pellet was resuspended in RPMI-1640. The resuspended cells were again subjected to centrifugation at 1200 rpm for 10 minutes. Supernatant was discarded and cell pellet (whole spleen cell suspension containing both leukocytes and erythrocytes) was resuspended in RPMI-1640.

3.2.2.2 Determination of spleen cell viability and concentration

10 μl of spleen cell suspension from the above step 3.2.2.1 and 90 μl of trypan blue were mixed thoroughly. A drop was taken from it and placed on glass slide to determine the viability. Dead cells take up the stain while live ones do not. A viability count of more than 80 per cent were used for immunizing inoculations. Cell density of the final suspension was determined by use of a hemocytometer observed under microscope (OLYMPUS VANOX; Olympus Optical Co. Ltd., Japan). The concentration was adjusted at $25X10^6$ white cells per ml with RPMI-1640.

3.2.2.3 Spleen cell inoculations for secondary (booster) immunization

Primed female mice were injected intraperitoneally with the male mice whole spleen cell suspension. Approximately 0.2 ml ($5x10^6$ white cells) per female mouse using disposable plastic tuberculin syringe (4G needle) was injected.

3.3 Collection of mice blood for antisera

Immunized female mice were bled from the tail at different times post skin transplantation and spleen cell inoculations as per the schedule shown in Table 1. The blood was pooled from different immunized female mice. The pooled and clotted blood was kept in the refrigerator at 4^oC for 24-48 hours for separation of the serum. Serum was separated and stored at -20^oC till further use in immunofluorescence assay.

3.4.1 Preparation of spermatozoa

Semen preparations of sexually mature cow bull and buffalo bull were used for the study. Semen samples were transferred to the laboratory and were immediately observed under light microscope for acceptable quality as judged by sperm morphology and motility. Each sample was subjected to centrifugation at 1000 rpm for 5 minutes. The supernatant was discarded and pellet resuspended in RPMI-1640.

3.4.2 Determination of spermatozoa viability and concentration

For viability, one drop of the spermatozoa suspension and five drops of eosin and nigrosin stain were placed on a glass slide and mixed well by blowing air. A smear was made and dried for 30 seconds to 1 minute. Slide was examined under light

microscope for viability. Dead cells take up the stain and live cells do not. Preparations with more than 80 per cent viability were used for further studies.

For adjusting the concentration, the suspension was charged into haemocytometre and observed under microscope (OLYMPUS VANOX; Olympus Optical Co. Ltd., Japan) and concentration adjusted at $2x10^6$ cells per ml with RPMI-1640.

3.5 Indirect immunofluorescence assay for detection of HY antigen positive spermatozoa

Goat anti-mouse polyvalent immunoglobulin (IgG, IgA, IgM) FITC conjugate, product No. F-1010, Sigma, USA was used.

The test was carried out as shown in the text below:

	Tube A (Test sample)	Tube B (Control sample)
1) Spermatozoa suspension in RPMI-1640	200 µl	200 µl
2) Mouse anti-HY serum*	20 µl	-
3) Diluent (RPMI-1640)	-	20 µl

Incubation on ice (for 30-40 min.) with occasional mixing

* Final testing dilution was 1:10. In some experiments as shown in results, a further four fold higher dilution (i.e. 1:40 final dilution) of the serum giving positive fluorescence was also tested.

First wash with 4-5 ml RPMI-1640 at 1000 rpm for 5 minutes. Supernatant discarded and pellet resuspended in RPMI-1640.

Second wash at 1000 rpm for 5 minutes with 4-5 ml RPMI. Supernatant discarded and pellet resuspended in 200 µl RPMI-1640.

To the washed spermatozoa suspension (200 µl volume), 20 µl of antimouse polyvalent immunoglobulin FITC conjugate was added in both the tubes. From this step onward, the reactions were carried out in dark. The spermatozoa suspension was incubated for 30 minutes on ice in dark. After incubation, washing was given at 1000 rpm for 5 minutes with ice cold RPMI-1640. Finally the pellet was resuspended in 100 µl RPMI-1640 for further use.

3.6 Fluorescence microscopy

Ten microliters of the spermatozoa suspension prepared as above was placed on a clean glass slide, covered with a clean coverslip and observed under UV microscope (Olympus AHC-RFCA; Olympus, Japan).

Table 1 IMMUNIZATION PROTOCOL

S.No.	Immunization	No.of grafts/ amount of immunizing inoculations (per female mouse)	No.of female mice	No. of male donors for skin grafts or spleen cell preparation	Days to rejection/ Result	Collection of blood for preparation of antiserum(days post transplantation or spleen cell inoculation)
A.	**Primary Immunization**					
1.	First skin transplantation	3 grafts	10	12	18-20 days	20 days - Serum I
B.	**Secondary/Booster Immunization**					
1.	Second skin transplantation	2 grafts	8	6	8-10 days	10 days - Serum II
2.	Spleen cell injection	0.2 ml($5x10^6$ cells) (intra peri-toneally)	7	4	-	7 days - Serum III
3.	Third skin transplantation	One graft	5	2	6-7 days	7 days - Serum IV
4.	Spleen cell injection	0.2 ml($5x10^6$ cells) (intra peri-toneally)	3	2	-	7 days - Serum V

Fig. 1 : Circular pieces of skin grafts (size 40-55 mm^2) punched out from the tail of C57BL/6 inbred strain male mouse for transplantation onto the female mice.

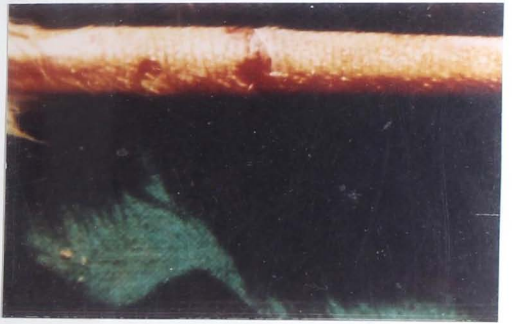

Fig. 2 : Preparation of skin sites at thorax region of the receipient C57BL/6 inbred strain female mouse for planting skin grafts from the syngeneic male mice.

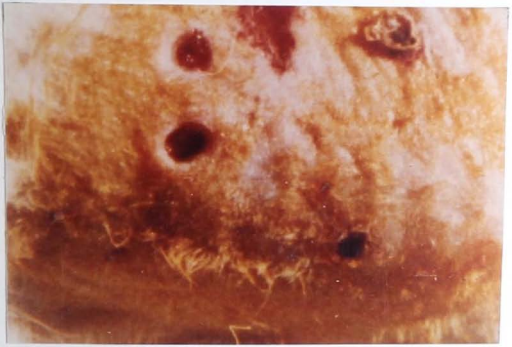

Fig. 3 : Group of C57BL/6 inbred strain female mice with skin grafts planted onto thorax region for immunization.

CHAPTER 4

RESULTS

Studies were carried out on spermatozoa of cow and buffalo bull using anti-HY antisera produced in C57BL/6 inbred strain mice.

4.1 Production of antisera to HY antigen

Skin transplantation and rejection of skin grafts

A group of 10 female mice of C57BL/6 inbred strain were used for transplantation of skin grafts from male mice.

The day the graft got totally dislodged was scored as the day of rejection.

The primary skin grafts were rejected between 18 and 20 days.

Rejection of secondary (first booster immunization) skin grafts were seen between 8 and 10 days.

Rejection of subsequent skin grafts (third booster immunization) were seen between 6 and 7 days.

Rejection of skin grafts was an indication of primary and booster immunization (Table 1).

Serum collection

Time schedule of sera collected at various time intervals post immunization is outlined in Table 1. These sera were labelled as Serum I, Serum II, Serum II, Serum IV and Serum V, collected respectively after primary and first, second, third and fourth booster immunization.

4.2 Immunofluorescence studies

Serum collected from immunized female mice post skin transplantation or spleen cell inoculation were tested for

antibody activity to spermatozoa using immunofluorescence test as described in section 3.5.

(1) First serum preparation collected 20 days post primary skin transplantation (Serum-I, Table 1) showed no antibody activity as there was no fluorescence of the spermatozoa

(2) Second serum preparation collected 10 days post second skin transplantation (Serum-II, Table 1) gave no fluorescence of the spermatozoa or occasionally very few spermatozoa gave fluorescence under different microscopic fields

(3) Third serum preparation collected 7 days post second booster immunization with intraperitoneal spleen cell inoculation (Serum-III, Table 1) gave similar results as that of Serum-II preparation. Either very few spermatozoa showed fluorescence or there was no fluorescence of spermatozoa

(4) Fourth serum preparation collected 7 days post third booster immunization with skin transplants (Serum-IV, Table 1) gave fluorescence of spermatozoa (Table 2, Table 3).

In immunofluorescence assay on buffalo spermatozoa with Serum IV, of the total 90 spermatozoa counted, number of fluorescing spermatozoa was 49. Percentage of fluorescing spermatozoa was approximately 54% in buffalo (Table 2). Control samples without antiserum did not show any fluorescence. In immunofluorescence assay on cattle spermatozoa with Serum-IV, of the 88 spermatozoa counted, the number of fluorescing spermatozoa was 46 (Table 3). Percentage of fluorescing spermatozoa was approximately 52% in cattle (Table 3). Control samples without antiserum did not show

any fluorescence.

After determining the positivity of Serum-IV, experiment with four fold higher dilution (1:40) was set up of the previously tested Serum-IV at 1:10 dilution. At this higher dilution, the immunofluorescence was negative in both cattle and buffalo spermatozoa

(5) Fifth serum preparation (Serum-V, Table 1) was collected 7 days post fourth booster immunization using intraperitoneal inoculation with spleen cells. There was fluorescence of spermatozoa (Fig.5).

In immunofluorescence assay on cattle spermatozoa with Serum V, of the total number of 95 spermatozoa counted, number of fluorescing spermatozoa was 49. Percentage of fluorescing spermatozoa was approximately 51.5% in cattle (Table 4). Control preparation of spermatozoa without antiserum did not show any fluorescence.

Immunofluorescence assay was conducted on spermatozoa using four fold higher dilution (1:40) of the positive Serum-V (1:10 dilution). In the samples with 1:40 dilution, there was no fluorescence

In spermatozoa preparations, there was clumping of spermatozoa, so only those spermatozoa were counted (both in non-fluorescing and fluorescing phase) which were lying as single cells to avoid errors. Clumping in the spermatozoa preparation was an obstacle to include clumped spermatozoa in the counts.

The pattern of fluorescence was that in general the whole spermatozoa including the tail showed fluorescence, (Fig.6) but the fluorescence was more intense on the acrosomal cap of the head of spermatozoa.

Table IMMUNOFLUORESCENCE ASSAY ON BUFFALO SPERMATOZOA(SERUM IV)
(Dilution 1:10)

Total No.of spermatozoa (per microscopic field)	No. of fluorescing spermatozoa (per microscopic field)
9	5
13	7
18	10
8	4
9	5
11	6
6	3
16	9
90	49

Percentage of fluorescing spermatozoa = (49/90)x100 = 54.44%

Table IMMUNOFLUORESCENCE ASSAY ON CATTLE SPERMATOZOA (SERUM IV)
(Dilution 1:10)

Total No.of spermatozoa (per microscopic field)	No. of fluorescing spermatozoa (per microscopic field)
13	9
11	5
15	8
9	5
16	7
10	5
14	7
88	46

Percentage of fluorescing spermatozoa = (46/88)x100 = 52.27%

Table IMMUNOFLUORESCENCE ASSAY ON CATTLE SPERMATOZOA(SERUM V) (Dilution 1:10)

Total No.of spermatozoa (per microscopic field)	No. of fluorescing spermatozoa (per microscopic field)
9	4
11	6
12	6
8	4
7	4
14	8
18	10
16	7
95	49

Percentage of fluorescing spermatozoa = (49/95)x100 = 51.58%

Fig. 4 : Spermatozoa preparation (cow bull) as seen under non-fluorescing phase of microscope used in the study.

Fig. 5 : Fluorescing spermatozoa in the immunoflourescene assay using Serum-V and cattle spermatozoa. The whole body of spermatozoa shows fluorescene with more intense fluorescene on acrosomal cap.

Fig. 6 : The differential pattern of fluorescene with more intense fluorescence on acrosomal cap.

CHAPTER 5

DISCUSSION

Producing offsprings of pre-determined sex, will have an immense potential in the livestock industry. There are at present two potential ways of influencing the sex ratio. These are separation of sperm into viable fractions of X and Y chromosome bearing sperms and use for artificial insemination (van Vliet et al., 1989) or by identification of the sex of pre-implantation embryos followed by embryo transfer(van Vliet et al., 1989). The essential pre-requisite is availability of standard immunological and non-immunological markers for embryo and sperm sexing. There are considerable number of research studies reporting development of immunoprobes i.e. antisera and even monoclonal antibodies to HY antigen (male specific antigen) which have been used for embryo and spermatozoa sexing.

The present study was conducted to raise antisera to male-specific antigen(s) using inbred mice strain and study its reactivity with cattle and buffalo spermatozoa using immunofluorescence assay with the aim to develop immunoprobes for sexing spermatozoa of dairy cattle and buffalo using anti-mouse HY antisera system. The present study was an attempt to produce the probe/antisera by ourselves under Indian conditions because such probes are not available commercially for use.

C57BL/6 inbred strain mice, both male and female, were used in the present study as this strain is considered to be the best responder for immune response to HY antigen (Eichwald and Silmser, 1955, 1958; Billingham et al., 1965 and Silvers, 1968).

Antiserum to male specific antigen was produced by primary immunization of female mice with syngeneic male skin grafts followed by repeated secondary immunizations with either skin transplantation or intra-peritoneal inoculation of syngeneic male spleen cells. Sera collected at different times post immunization were tested in the indirect immunofluorescence assay (IFA) on spermatozoa using goat anti-mouse polyvalent immunoglobulin FITC conjugate.

Absence of immunofluorescence of spermatozoa with sera from early immunization, and positive immunofluorescence from sera after booster immunizations suggests that antibody activity developed only after sufficient boosting.

The immunofluorescence reaction was considered to be specific on the basis that (i) the control samples treated only with anti-mouse immunoglobulin FITC conjugate did not show fluorescence, (ii) the absence of immunofluorescence of spermatozoa with female mice sera from early immunizations (Serum I, II and III) could be considered retrospectively as control.

The immunofluorescence studies with the antisera samples (Serum-IV and Serum-V) obtained post third and fourth booster immunization respectively that give positive reaction at 1:10 dilution were also tested at four fold higher dilution (1:40 dilution) of the sera. With the 1:40 dilution, there were no fluorescing spermatozoa. The results show that there were only low titers of antisera.

Booman et al. (1989) reported that only a low percentage of mice have good antibody response to HY antigen and antisera are usually low affinity and low-titered.

Simpson (1983) reported that HY belongs to a class of weak transplantation antigens characterized by an inability to elicit responses under many conditions.

Bradley and Heslop (1985a) introduced a method for the production of high titer HY antisera in BN and HS inbred strain rats. Antisera with titers as high as 1:200 to 1:400 were obtained by transplanting syngeneic male skin into the spleen of female recipient. This method for production of high titer antisera could not be tried in our studies as this method was difficult to accomplish on mice due to smaller body size of mice than rats. Secondly, sufficient number of BN and HS inbred rats, due to very low breeding efficiency, could not become available for our study.

In our results pattern of fluorescence observed was that in general the whole spermatozoa, including tail, showed fluorescence. The fluorescence was more intense in the acrosomal region. It seems there is regional differentiation of HY antigen, as fluorescence was more intense on the acrosomal region of the sperm surface. Koo et al. (1981) using mice spermatozoa showed that the fluorescence specifically associated with HY antigen was more intense on the acrosomal cap of the head, while little or no fluorescence was present on tail of mice spermatozoa.

Iyer et al. (1989) reported that HY antigen was localized on the acrosomal membrane of mouse epididymal and washed ejaculated human spermatozoa using indirect immunofluorescence technique.

Bradley (1989) reported that HY antigen was located on both the post-acrosomal region of the head and the midpiece of the flagellum of cow bull spermatozoa.

Ali et al. (1990) conducted studies on bovine spermatozoa using monoclonal HY antibodies and showed that fluorescence of sperm was most intense in their post acrosomal region.

Cross-reaction of murine HY antisera with cattle and buffalo spermatozoa supports the well established fact that HY antigen is highly conserved. Murine HY antisera cross-reacts with HY antigen in both vertebrates (Silvers and Wachtel, 1977; Wachtel, 1983) and invertebrates (Wachtel et al., 1975).

The findings of Ali et al. (1990) indicated that the available monoclonal antibody to HY antigen produced from mice ascites fluid binds to approximately half of bovine spermatozoa as a result of haploid gene expression of Y-chromosome bearing spermatozoa. There are some reports unable to support the haploid expression on HY antigen on Y-chromosome bearing spermatozoa. These reports suggested that sertoli cells and not germ cells synthesize HY antigen (Brunner et al., 1984; Zenzes et al., 1988) and that spermatids have the ability to bind HY antigen (Koo et al., 1979) and spermatozoa passively become HY positive regardless of sex chromosomal composition.

Ali et al. (1990) showed that HY antigen appears to be expressed on the plasma membrane of haploid Y-chromosome bearing cow bull spermatozoa.

Results of the present study showed that detection of male specific protein (HY antigen) was approximately on 50 per cent of cattle and buffalo spermatozoa, which suggests that the fluorescence positive ones could be Y-chromosome bearing spermatozoa and the non-fluorescing ones would be X-chromosome bearing spermatozoa. However, the present study did not prove it and is an important question which needs to be resolved in future studies. This will require sorting out the fluorescing and non-fluorescing spermatozoa using fluorescence activated cell sorter (FACS) followed by using the sorted preparation for artificial insemination and confirmation by conception results. DNA analysis of the sorted spermatozoa using Y-specific probes i.e. molecular biology approach will be informative.

The present study results will serve as pre-requisite to further develop monoclonal antibodies to HY antigen so that large amounts of anti-HY immunoprobes having high specificity become available for further use in sorting bovine X and Y chromosome-bearing spermatozoa and use in artificial insemination.

CHAPTER 6

SUMMARY

Producing offsprings of pre-determined sex, will have an immense potential in the livestock industry. Sex pre-selection can be done: (i) before fertilization, i.e., spermatozoa sexing followed by artificial insemination (AI); (ii) after fertilization, i.e., embryo sexing followed by embryo transfer (ET). In the present study, antisera to male specific antigen(s) was produced in female mice of C57BL/6 inbred strain using skin transplantation of skin grafts from the syngeneic male mice as primary immunization followed by several booster immunizations with skin grafts or spleen cell inoculations from the syngeneic male mice. The syngeneic male and female mice are identical at the MHC except for male specific antigen (HY antigen) and rejection of grafts of male tissue on the syngeneic females is due to immunological response to the histoincompatibility of male specific antigen (i.e. the HY antigen). Amongst various inbred mice strains, the C57BL/6 inbred strain is known to be the best responder for immune response to HY antigen. Reactivity of antisera with cattle and buffalo spermatozoa using immunofluorescence assay was studied with an aim to develop immunoprobes for sexing spermatozoa of cattle and buffalo. For immunization with skin graft transplantation, skin grafts from the tail of male mice were punched out (40 to 55 mm^2 size) using grafting punch, and grafted onto the reciprocal punched out skin sites of thorax region of female mice. For preparation of the skin grafts for transplantation, both the donor male mice and the

recipient female mice were anaesthetized using ketamine (0.2 ml/20 g mouse, intra-muscularly) followed by maintenance of anaesthesia with ether. The grafts of first skin transplantation experiments got rejected between 18 and 20 days post transplantation, second skin transplantation between 8 and 10 days and the third skin transplantation between 6 and 7 days post-transplantation, indicating immunization protocol was effective. At two intervals i.e. second and fourth booster immunizations were done with intraperitoneal inoculation of spleen cells, instead of skin grafts, from male mice into the female mice. Serum was separated from the blood collected from immunized female mice at different occasions of the immunization. These were labelled as Serum-I, II, III, IV and V collected respectively after primary and first, second, third, fourth booster immunization.

Antibody activity of sera was tested by Indirect Immunofluorescence Assay (IFA) using goat anti-mouse polyvalent immunoglobulin FITC conjugate. Sera from early immunizations (i.e. Serum-I, Serum-II and Serum-III) did not have antibody activity as there was no fluorescence of spermatozoa (Serum-I) or only 4-8 per cent with Serum-II and III, respectively. Fourth and fifth serum were positive for antibody activity and the percentage of fluorescing spermatozoa was approximately 54% in buffalo spermatozoa and 52% in cattle spermatozoa. Immunofluorescence on spermatozoa was observed only after hyperimmunization. Pattern of fluorescence observed was that in general the whole spermatozoa, including tail, showed fluorescence. The fluorescence was more

intense on the acrosomal region. It seems there is regional differentiation of HY antigen, as fluorescence was more intense on the acrosomal region of the sperm surface.

The immunofluorescence studies with the antisera samples obtained post third and fourth booster immunization respectively (Serum-IV and Serum-V) that give positive reaction at 1:10 dilution were also tested at four fold higher dilution (1:40 dilution) of the sera. With the 1:40 dilution, there were no fluorescing spermatozoa. The results showed that antibody titres were low in the antisera.

The immunofluorescence reaction was considered to be specific on the basis that (i) the control samples treated only with anti-mouse immunoglobulin FITC conjugate did not show fluorescence, (ii) the absence of immunofluorescence of spermatozoa with female mice sera from early immunizations (Serum I, II and III) could be considered retrospectively as control.

Cross-reaction of murine HY antisera with cattle and buffalo spermatozoa supports the well established fact that HY antigen is highly conserved.

Results of the present study showed that detection of male specific protein (HY antigen) was approximately on 50 per cent of cattle and buffalo spermatozoa, which suggests that the fluorescence positive ones could be Y-chromosome bearing spermatozoa and the non-fluorescing ones could be X-chromosome bearing spermatozoa. However, the present study did not prove it and is an important question which needs to be resolved in future

studies. This will require sorting out the fluorescing and non-fluorescing spermatozoa using fluorescence activated cell sorter (FACS) followed by using the sorted preparation for artificial insemination and confirmation by conception results. Secondly, DNA analysis of the sorted spermatozoa using Y-specific probes i.e. molecular biology approach will be informative.

The present study results will serve as pre-requisite to further develop monoclonal antibodies to HY antigen so that large amounts of anti-HY immunoprobes having high specificity become available for further use in sorting bovine X and Y chromosome-bearing spermatozoa and use in artificial insemination.

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Annexure-I

The following additional information should be read with the Results at Page 35 and also the Material and Methods at page 29.

A. Testing of Serum I, II, III, IV and V for antibody activity was done with fresh semen samples from the same bull. The Serum IV and V gave optimal antibody positive reaction in the IFA. The Serum IV and V being two different samples were tested at two different occasions and represent two different reproducible experiments since the results were similar with both the positive sera using fresh semen samples from the same bull.

B. In subsequent three repeat experiments on Serum IV and V (Antibody positive sera), liquid chilled semen samples were used in place of fresh semen samples obtained from the same animal kept by the Department of Veterinary Gynaecology. For preparation of spermatozoa, such samples needed extra washings and centrifugations to remove the egg yolk citrate used as extender/diluent in the preserved liquid chilled semen samples. The pattern of fluorescence with such preparations was not characteristic of membrane fluorescence in the IFA. The fluorescence was rather internalized in the spermatozoa (Refer to Annexure I, Fig.7). It was observed that the percent dead sperms was also higher than that in the fresh semen samples. One of the possible explanation could be that the dead spermatozoa might have taken up and internalized the fluorescent dye. However, this may not be the only possible explanation, because though the number of dead spermatozoa was higher in the liquid chilled semen samples than in the fresh semen samples, still the number of fluorescing spermatozoa did not correlate with the number of dead spermatozoa.

Fig.7: Pattern of fluorescence of spermatozoa with liquid chilled semen used in the study.