3. MATERIALS AND METHODS

3.1 Material required for the production of blood group reagents

The animals used in this study belonged to the Red Sindhi Sahiwal, Tharparkar, Brown Swiss, Brown Swiss crosses with Red Sindhi and Sahiwal breeds, stationed at National Dairy Research Institute, Karnal; Kankrej herds maintained at Institute of Agriculture, Anand and Cattle Breeding Farm, Chaarodi, Ahmedabad; Jersey Breed stationed at Central Cattle Breeding Farm, Hessarghatta; Holstein Friesian, Red Sindhi x Jersey and Red Sindhi x Red Dane crosses maintained at State Livestock Farm, Hessarghatta and Haryana animals stationed at Haryana Agricultural University, Hisar.

The blood group reagents were produced by using iso- and hetero-immunization methods. Suitable pairs of animals were selected for iso-immunization from Tharparkar, Red Sindhis, Sahiwals and Brown Swiss crosses. The blood types of some of the Tharparker animals were available from the records of the tests done at the Copenhagen laboratory* in 1962 and a few pairs of animals of other breeds were selected on the basis of reactions for certain hetero-immune buffalo blood group reagents which were available.

* through the courtesy of Professor Moustgaard
Twenty eight pairs of cows and heifers were selected as donors and recipients on the basis of minimum differences in number of antigens present on the red cells of these pairs.

Ten healthy rabbits, negative for naturally occurring antibodies in cattle, were used as recipients for the production of heteroimmune sera.

Materials such as syringes, hypodermic needles, transfusion bottles, rubber adapters, sterilising equipments, refrigerators, anticoagulant (sodium citrate 2 per cent with 0.5 per cent sodium chloride made up to 100 ml distilled water) were also used during the work.

Special test tube racks made of aluminium and of the size of 21 x 5 cm x 6 cm to accommodate two rows of ten Kahn test tubes each were used. Haemolytic test sheets for recording the reactions (designed by Dr. L.C. Ferguson) were used.

3.2.0 Methods for blood typing

Antibodies were produced in such animals against certain blood factors (antigenic factors) whose blood types did not possess those antigens. The practical blood group serology was based on this fundamental concept. The blood types of donor and recipient animals used in this study were obtained based on haemolytic tests, using both cattle and buffalo heteroimmune reagents. Based on blood types, the donors and recipients were chosen in such a way that the donor cells possessed only one or two known antigenic factors not possessed by the recipient animals.
3.2.1 Collection of blood for immunization

The donor animals were restrained in a trevis and the jugular vein was raised by pressing with the thumb. Taking due antiseptic precaution, the vein was punctured with a sterilised needle with a sterilized rubber tube attached to it. The free end of the rubber tube also carried a sterile hypodermic needle. The blood from the donor animals was collected through the free end of needle into previously evacuated transfusion bottles which contained 100 ml of sterilized anticoagulant solution.

When sufficient quantity of blood had flowed (usually 300 ml) the collection of blood from the animal was stopped. The bottle was inverted gently to mix with the anticoagulant solution.

Blood samples from both the donors and recipients were collected in glass tubes containing 5 ml of anticoagulant citrate solution for conducting the haemolytic tests. Small quantities of blood samples were also collected from all the animals in sterile dry glass test tubes for obtaining blood sera to be used in the above test. All the blood samples collected in glass tubes containing anticoagulants were preserved in the refrigerator at 4°C until further use. The separation of blood sera was hastened by keeping the glass tubes containing clotted blood at 37°C in water bath and the tubes were transferred into a refrigerator after separation of the clot from the sides of the glass tube.
The sera thus obtained were centrifuged at low speed to remove the red cells from them.

3.2.2. Production of blood group reagents

Iso-Immunization

50 ml of citrated blood of the donor, collected earlier was injected into selected recipients intravenously as the first dose and subsequently 20-25 ml of the donor's blood was injected at weekly intervals for 6-8 weeks depending upon the reaction. Before injecting the donor's blood each time about 10-15 ml of blood samples from recipient animals were collected separately into glass tubes containing 5 ml of sodium citrate (anticoagulant) and dry tubes. The serum obtained from every recipient before the first injection, was tested for the presence of naturally occurring antibodies and subsequent serum samples obtained at weekly intervals, were utilized to find out the titre of the serum.

Invariably, during second injection, most of the animals had symptoms of anaphylactic shock varying from tremors and salivation to very unsteady gait and fits. Such animals got an injection of 5 ml of 1 in 1000 adrenaline intramuscularly and the symptoms ceased after 10-15 minutes. During subsequent injections, the animals showed only mild anaphylactic reactions.

A total of 28 animals were immunized in three batches to produce 12 isoimmune reagents.
In the first instance, the titres of the sera produced were not satisfactory. Reimmunization of the same recipients was done after a lapse of 2-3 months.

For reimmunization, the same techniques were followed except that the volume of donor's blood injected was much less, i.e., 25 ml in the first injection and 15-20 ml in subsequent injections. By about the fourth injection, the highest titre was produced in these animals.

**Heteroimmunization**

Red cells from few donor animals were washed repeatedly with normal saline (at least three times) till the supernatant solution was free from even traces of serum.

A batch of 10 rabbits, which were negative for naturally occurring antibodies, against cattle red cells were used for heteroimmunization. The external marginal ear veins were used for injecting the 50 percent red cell suspension in normal saline.

First injection of 0.2 ml of cells was carefully introduced intravenously. The injections were repeated on alternate days with a gradual increase in the volume of cell suspension till a volume of 1.5 ml of 50 percent red cells of donor cattle was injected or till the titre was sufficiently high for harvesting the serum. Everytime before the introduction of donor cells into marginal ear vein of rabbits a small quantity of blood was collected and the sera obtained were used for estimating the titre.
Four to seven days after the last injection, the rabbits were bled sufficiently to produce enough sera of high titre. The heteroimmune sera thus produced were stored frozen at about -20°C till it was fractionated.

3.2.3 Analysis of serum

The sera collected every week from the recipient cattle in the case of isoimmunization and on alternate days from the immunized rabbits were subjected to haemolytic tests in order to assess the titre by testing against different cattle red cells.

Red blood cells from 20 cattle including the cells of all donor animals in use were chosen for the haemolytic tests. One ml each of cattle blood was taken separately in glass or polystyrene centrifuge tubes (Permanent marks had been made on the tubes to indicate 0.3, 1.0 and 10.0 ml volumes). The volumes were made up to 10 ml with normal saline. The tubes were shaken and centrifuged for five minutes at 2000 r.p.m. The supernatant fluids were removed and the processes were repeated till the cells were free from the sera. The cell volumes were reduced to 0.3 ml mark and by the addition of normal saline up to 10.0 ml mark a three percent suspension of different cell samples was obtained.
The special test tube racks were arranged for finding out the titre. The number of racks in the horizontal row depended on number of cells used. The number of racks in the vertical rows depended on number of sera tested in doubling dilution. In addition, one row of racks at the far end was meant for (1) Saline and (2) Complement controls. Kahn test tubes were used for studying the titre by haemolytic technique.

Two drops (0.1 ml) of serum (approximate dilution) were placed in each tube in the horizontal rows. In the penultimate row two drops of normal saline per tube and in the last row three drops of normal saline per tube were placed.

Red cell suspensions prepared from individual animals were dropped in vertical rows at the rate of one drop per tube. Ultimately each serum in a horizontal row would contain all the blood cells used in separate tubes. The racks were shaken and left for 15 minutes. Then one drop of undiluted rabbit serum (fresh rabbit sera free from naturally occurring antibodies against cattle red cells) was added in every tube except the tubes in the last horizontal row. All the racks were shaken and the time was recorded on the haemolytic test sheets specially designed for this purpose.

After half an hour, the racks were shaken and the extent of haemolysis was recorded with a lead pencil according to the following scale:
(0) - All cells were intact, no light passed through the suspensions.

+ - About 50 percent of the cells were lysed; much light passed through the fluid.

+ - All cells were lysed. Liquid was transparent and sparkling red.

One hour after first reading the second reading was taken and recorded in ink. After the second reading the racks were shaken. The third reading was taken 90 minutes after second reading and recorded in red pencil.

The scale used for second and third readings was as follows:

0 - All cells were intact and settled at the bottom, supernatant was clear, with no trace of colour.

0+ - Almost all cells were intact and settled at bottom; supernatant was slightly reddish.

Tr. (Trace) Nearly all cells were intact, and settled at bottom, supernatant was reddish coloured.

1 - Most cells were intact and settled, supernatant was red.

2 - More than 50 percent of cells were lysed, the unlysed cells settled at the bottom in the form of a small button or ring.

3 - Nearly all cells were lysed, supernatant was bright red; when tube was shaken, the liquid became cloudy.

4. - All cells were lysed; liquid was sparkling red, and remained so even after the tube was shaken.
3.2.4 Absorption technique

The sera obtained from iso and hetero-immunization were considered to be polyvalent. In the case of isoimmune sera it was less polyvalent, presumably because the difference in the antigen components within a species being due to differences between inherited variations within that species. However, in the case of heteroimmune sera, the immunized rabbits produced not only the antibodies due to differences in the antigenic component between two donors, but also produced species specific antibodies capable of reacting against almost all the cattle red cells. Hence it was more difficult to make the heteroimmune sera monospecific than was the case with isoimmune sera.

From the results of haemolytic tests, it was seen whether all the cells which possessed a particular antigenic factor had reacted. If one or more of such cells did not show any reaction, then it was decided that antibodies against that antigenic factor were not present in the serum. The cells in the case of isoimmune sera showing weak reactions were first chosen for absorption. Since all cells were reacting with heteroimmune sera, initially all cells, one at a time, were used for absorption.

Trial absorptions were carried out with 5 ml of a properly diluted serum taken in a polythene centrifuge tube (the dilution was calculated by multiplying the highest titre of the serum expressed as a fraction by eight, as
recommended by Lazear and Ferguson (1953). The cells chosen for absorption were washed thrice in saline. Suitable volumes of packed cells to represent 10, 15 and 20 percent of serum taken were pipetted out into the serum, shaken well and kept for 30 minutes with occasional shaking. Then the contents were centrifuged and supernatant removed and saved. The cells used for absorption were discarded.

A careful scrutiny of haemolytic test with serum absorbed using blood cells from a single individual was done and any weakened reactions when compared to unabsorbed serum test were noted. In addition to first cells used, the second series of cells showing weak reactions were added, and the entire process was repeated. Absorptions, testings and additions of next series of cells were done till the final absorbed serum was monospecific or passed unity test. For finding out monospecificity, the serum under test was absorbed with each of the cells showing reaction. If after each absorption, the resulting serum showed no reaction with any of the cells in the haemolytic test, this was taken as evidence that the serum contained only antibodies against a particular antigenic factor, and was considered to be monospecific.

If the absorption was not successful, several combinations of cell concentrations and dilutions of serum and also different absorbing cells were tried until the serum containing a unit antibody was obtained.
3.2.5 Blood typing

Blood samples from 1215 animals (291 Tharparkar, 253 Sahiwals, 86 Red Sindhi, 129 BxS + 34 B x R, 114 Kankrej, 69 Mariana, 75 Jersey, 59 Holstein, 20 Red Sindhi Jersey cross, 14 Red Sindhi Red Dane cross) were collected for testing with the 14 monospecific reagents (twelve produced by iso-immunization and two by hetero-immunization). The procedure described earlier was used for the haemolytic tests.

3.2.6 Parentage tests

For studying the parentages, only the dairy herds maintained at National Dairy Research Institute, Karnal were utilized. The animals belonged to Red Sindhi, Sahiwal, Tharparkar and crosses of Red Sindhi and Sahiwals with Brown Swiss breed. In the present case, the dams were bred by Artificial Insemination to different sires of the same breed in the same or two consecutive heat periods. Some of the calves born to these inseminations had doubtful paternity and the identity of sires was not recorded. In all, paternity of nine Brown Swiss crosses, five Red Sindhis, 15 Sahiwals and 16 Tharparkar animals born during the period from 1962 to 1969 were not recorded (Table 3). Further, the accuracy of the pedigree records of the above herds was also tested. One hundred and sixteen complete sire-daughter-off-spring sets were investigated by comparing the blood factors, as well as haemoglobin, transferrin and albumin types of these animals (Table 4).
3.2.7 Genetic studies

Alleles present at blood group loci can be determined by the inheritance of the blood factors through the sire family studies. However, it is not so easy to find out the associations between various blood factors and the alleles that exist at a particular locus because of the smallness of the sire families. Hence an alternative method of establishing the associations between various blood factors by chi square values was followed. Large number of random animals from a single population have been blood typed. The chi square values obtained by the $2 \times 2$ contingency tables were utilized to study the significant associations between various blood factors (Table 5).

In the present study, the blood type information of six sire families were also available. From the blood type information of the offsprings of different sires, the alleles operating at different blood group loci (Table 6) were estimated.

3.3 Biochemical typing of haemoglobins

The serum free red cell suspension available after blood typing was centrifuged at 2000 r.p.m. for five minutes and the supernatant was discarded. The packed cells were lysed by adding equal quantity of distilled water and stored frozen at about $-20^\circ$C overnight. The haemolysate, thus obtained was adjusted to 10 per cent concentration of haemoglobin. The hydrolysed starch for the study of haemoglobin types was prepared as described by Smithies (1955) with modifications.
as detailed below to get good resolution.

**Hydrolysis of starch**

A water bath was maintained at the temperature of 38°C at least for two hours. Sixty ml of acetone (double distilled) was taken in a conical flask and to it 1.5 ml of hydrochloric acid (Analar sp. gr. 1.16) was added. It was kept in the water bath and 30 g of B.D.H. potato starch was added to acetone-acid mixture and mixed approximately at five minute intervals. At the end of 30 minutes, 30 ml of 18 percent sodium acetate solution was added to stop the hydrolysis of starch by the acid in the acetone mixture. The hydrolysate was filtered through a Buchner funnel and the starch was washed two to three times with one to two litres of glass distilled water. Then the starch was suspended in sufficient quantity of glass distilled water and kept overnight. Next day, it was washed once again with glass distilled water and finally with 100 ml of acetone to remove the moisture. This starch was spread over a clean filter paper and dried in hot air oven at 37°C and was used for electrophoretic study.

**Electrophoretic method**

First, the electrode vessel of electrophoresis apparatus (of standard make) was filled up with Tris-E.D.T.A.-Boric acid buffer of pH 8.9 (Gahne et al., 1960). This electrode buffer was prepared by adding 20.2 g of Tris hydroxymethylamino methane (Sigma 7-9) 2.0 g of E.D.T.A and 1.5 g of
Boric acid to one litre of distilled water. The same buffer diluted for 10 times, was used as gel buffer. Next haemolysates were subjected to horizontal starch gel electrophoresis in gels made with 72 g hydrolysed starch in 120 ml of diluted gel buffer. It was necessary to alter the amount of starch slightly when shifting to new starch lots.

In order to make uniform gels, 2/3 of the volume of the gel buffer was heated to 100°C. and transferred rapidly to the starch suspended in 1/3 of the volume of the gel buffer. The resulting viscous material was shaken vigorously over the flame for about 15 seconds, degassed for about a minute by applying a vacuum pump to the flask and then poured into the two gel trays (Each tray had interior dimensions of 17.0 cm x 8.0 cm x 0.3 cm) and they were allowed to set and cool at room temperature.

After cooling, slots were made two cm from one end of the gel tray. Each slot was about half cm in length and in every plate not more than nine samples were inserted.

Whatman No. 3 mm. filter paper was cut into sizes of 0.5 x 0.3 cm. A filter paper insert was dipped in every one of the haemolysates and it was pressed on a filter paper to remove the excess fluid and the insert was carefully put into a different slot. Gel plates, after insertion of different samples, were kept inside the electrophoresis cell after covering with glass plates to avoid evaporation and the gel
ends were connected with filter paper bridge to make the electrical circuit complete. The power pack was switched on and was allowed to run for 10 minutes. At the end of this period, the power pack was switched off and the filter paper inserts were removed. It was again restarted and a voltage of 110 volts and 12 m.a. current per plate was applied for two hours. At the end of this time or when the haemoglobin bands travelled 6 cm from cathode end, the power supply was cut off. The gels were cooled and haemoglobin types were read directly. In the case of haemoglobin, the bands were easily visible and hence no staining was needed. However, for records, few gel plates were stained by means of (1 percent) Amido Black solution and was allowed to act for one minute and then destained by repeated treatment with a washing solution made out of Methanol: acetic acid: water in the ratio of 5:1:5.

3.4 Studies on blood serum proteins

3.4.1. Transferrins

The method used by Smithies (1955) for starch gel electrophoresis and in discontinuous buffer system of Poulik (1957) which was later modified by Kristjannson (1963) was employed.

Electrophoretic analysis of sera was carried out in gels made with 14 g hydrolysed (B.D.H.) starch in 120 ml of Tris buffer (1.73 g of Tris plus citric acid 0.85 g/litre) pH 7.6. The starch was suspended in 40 ml of buffer at room temperature and the remaining 80 ml was heated to 100°C before it was
added to the starch suspensions. The resulting viscous material was heated for a few more seconds and was shaken vigorously. It was degassed, poured into two gel plates and cooled to room temperature.

Next the serum samples were inserted in the gel plates as described earlier in the haemoglobin study. Gel plates were covered by means of glass plates to avoid evaporation. The gels were connected to electrode chambers by means of filter paper bridge. The electrode chambers contained a buffer solution made up of 0.3 M boric acid and 0.1 M NaOH (pH 8.7). An initial voltage of 175 V was applied for 15 minutes after which the current was shut off. The paper inserts were then removed. Then the gels were covered once again with glass plates and the current was passed for a further period of 15 minutes after which the voltage was increased to 350 V. The electrophoresis was run until the brown borate boundary line migrated by about 8 cm. This was accomplished in about two hours from the time the electrophoretic system was switched on to high voltage. The gel plates were removed from the electrode chamber and cooled to room temperature. The edges of the gel were separated from the sides of gel tray using a sharp razer blade. The glass sheet bearing the gel was taken out from the gel tray. The gel was separated from the surface of the glass plate by passing a fine metallic wire. Finally, the glass plate
bearing the starch gel was held upside down over a staining tray at such an angle as to facilitate the gel to fall into the tray gently when the edges of the gel were lifted by means of the blade. In this method the gel was not sliced because the gel itself was sufficiently thin and protein bands were quite clear and prominently visible after staining with 1 percent Amino Black solution and repeated destaining with the washing solution. Invariably each gel plate carried a sample of known transferrin phenotype (TfAB) for accurate identification of the unknown transferrin types of cattle.

3.4.2 Albumins

The use of a gel buffer at acid pH was essential for detecting the heterogeneity of this serum protein. The analytical procedure followed was that described by Kristjannson, (1966) with suitable modification wherever necessary. Before undertaking the investigation of serum albumin variants, a sample of crystalline bovine albumin was run in starch gel with the serum samples and the mobility of the albumin was confirmed.

Sera obtained from the animals, were subjected to horizontal starch gel electrophoresis in gels made with 14 g of hydrolysed B.D.H. starch in 120 ml of a 0.01 M. Tris
(hydroxymethyl amino methane) - 0.004 M citric acid buffer (pH 6.3). To prepare a uniform gel, the starch was suspended in 40 ml of gel buffer at room temperature and the remaining 80 ml was heated to 100°C and transferred rapidly to suspension. The resulting viscous mass was shaken vigorously for about 15 seconds, degassed and then poured into two gel plates. The gels were cooled and the serum samples were charged. The rest of the procedure was similar to that described earlier in the study of serum transferrin by horizontal starch gel electrophoresis.

Gels were connected to the electrode chambers which contained 0.3 M boric acid - 0.1 M NaOH buffer solution (pH 8.7). An initial low voltage of 90 V was applied for 15 minutes after which the current was shut off and the paper inserts were removed. After covering the gels with glass plastes, the voltage of 90 V was continued for a further period of 75 minutes after removing the insert papers and the voltage was increased to 180 V thereafter. The electrophoretic run was continued until borate boundary migrated 8 cm from the insert line. The rest of the procedure was similar to that used for studying serum transferrin in cattle. Each gel plate contained a known serum albumin type as a reference and the serum samples were classified and recorded accordingly.
3.5 Associations between antigens and certain economic characters of cattle

The age at first calving, 305 days yield, lactation length and inter calving period for the first lactation records of animals were utilized for this study. The records of animals with less than 150 days and records following absorptions were omitted.

The breedwise data were put on punch cards (IBM) and the comparisons were made between the animals having particular antigen and with those not having that antigen. A 't' test for group comparisons was used to test the significance of these differences using the mathematical formula of Snedecor (1961) for small sample size. The formula used for 't' was as follows:

\[ t = \frac{(x_1 - x_2) \sqrt{n_1n_2(n_1+n_2-2)}}{(n_1+n_2)\bar{x}^2} \text{ for } n_1+n_2 - 2 \text{ d.f.} \]

where \((x_1 - x_2)\) provided the difference between the groups means and \(\bar{x}^2\) devoted the pooled sum of squares. The test of significance for the difference between groups means, in the case of samples larger than 30 the ratio difference of means became a normal deviate. The values, S.E. of difference thus obtained was referred to the value of normal probability integral where \(t_{0.05} = 2.96\). The results were read accordingly.