CHAPTER 3

MATERIALS AND METHODS
3. MATERIALS AND METHODS

A knowledge of the blood group picture of donor and recipient pairs would have helped the development of the desired type of blood group reagent. But no buffalo blood group reagents were available in any of the leading immunogenetic laboratories of the country. So, the recipient-donor pairs were selected at random out of the related (dam-daughter) and unrelated animals for the production of reagents utilizing the experimental buffalo heifers kept at N.O.R.I., Karnal. Various materials used in these studies are described as follows:

3.1. Bleeding for cells

Properly sterilized veterinary 18 gauge size needles (kept in spirit) were used for collection of blood samples from the jugular veins of the buffaloes. The area above jugular vein was properly sterilized with spirit and rubbed well before and after pricking the needle for bleeding.
Glass test tubes of variable sizes depending upon the purpose of bleeding (panel or absorption) were thoroughly washed, rinsed with distilled water and dried in the oven after adding a requisite volume of anticoagulant. After taking the samples the tubes were thoroughly shaken to mix the anticoagulant with the blood to avoid clotting of the samples. The samples were then transported to the laboratory for storage in the refrigerator. Blood samples, more than a week old, were not used, because it was observed that blood cells started hemolyzing after 5–6 days and there were chances of getting false results in the hemolytic tests.

3.2. Panel cells

Twenty buffalo heifers of about one year age were selected at random as the source of panel cells. These animals were used for routine bleeding for setting up the hemolytic tests. Ten ml of blood was taken every time for the panel cells at an interval of 6–7 days. All these panel animals were buffalo heifers and no male was included to avoid any chance of auction or surplus disposal.

3.3. Complement

Undiluted rabbit serum was used as complement. Twelve rabbits of normal health were selected for this purpose. These animals were checked for naturally occurring antibodies in their sera and the rabbits whose sera contained these antibodies were not included. The blood was collected weekly from the marginal vein of the left ear. The blood was allowed to clot at
room temperature and was then kept in the refrigerator overnight. Next morning the serum was centrifuged at 3000 rpm. After pooling the collection from different rabbits was used immediately or stored in the deep freeze at a temperature of -20°C. Only fresh complement was used in the tests as far as possible. On an average each rabbit gave 10-15 ml of blood at each bleeding thus giving 5-6 ml of complement.

3.4. Routine testing

For the routine testing of various antisera with the panel cells special aluminium racks of size 21x5x6 cm, accommodating twenty test tubes in two rows of ten each were used. Corning glass test tubes (70 mm in height and 10 mm in diameter) were used for lytic test. A special pipette fitted with small rubber bulb was standardized to deliver 20 drops per ml of the serum.

Depending on the number of sera to be tested and the number of cells to be used, racks were set up and fitted with the haemolytic tubes. Two drops of each serum were dropped in horizontal rows. One drop each of washed 3% cell suspension was dropped along the vertical rows. Then the racks were shaken and incubated for 15 minutes before adding one drop of complement.

First reading was taken half an hour after adding the complement and the second and third readings were taken after one and 1½ hour intervals respectively. The readings were made...
on the basis of degree of haemolysis as shown in the following scales.

**First reading**

<table>
<thead>
<tr>
<th>0</th>
<th>All cells were intact and no colouring of the supernatant, no light passed through suspension i.e. no lysis.</th>
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<tbody>
<tr>
<td>+</td>
<td>About 50 percent of the cells were lysed, much light passed through the suspension.</td>
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<tr>
<td>+</td>
<td>All cells were lysed.</td>
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**Second and third readings**

<table>
<thead>
<tr>
<th>0</th>
<th>All cells intact and settled at the bottom, supernatant clear, with no trace of colour.</th>
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<tbody>
<tr>
<td>Tr</td>
<td>(Trace) Nearly all cells intact and settled at bottom, supernatant reddish in colour.</td>
</tr>
<tr>
<td>1</td>
<td>Most cells intact and settled with reddish supernatant.</td>
</tr>
<tr>
<td>2</td>
<td>More than 50 percent of the cells lysed, the unlysed cells settled at the bottom forming a small ring or button.</td>
</tr>
<tr>
<td>3</td>
<td>Nearly all cells lysed, with bright red supernatant that becomes cloudy after shaking.</td>
</tr>
<tr>
<td>4</td>
<td>All cells lysed with sparkling red fluid which remains so even after shaking.</td>
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The laboratory temperature varied considerably throughout the period of studies and the temperature is known to affect the degree of haemolysis. In order to make the results comparable, the temperature in the laboratory was controlled at 23-30°C.

In all the tests two sets of controls were always included:

i) Complement control - In this test 0.1 ml of normal saline replaced the antiserum, cells and complement were added as usual. This was done to test the haemolytic activity of the complement, if any.

ii) Saline control - Normal saline being used for cell washing, diluting antisera, was put to test by adding one drop (0.05 ml) of cell suspension to 3 drops (0.15 ml) of saline. This test assured the stability of the erythrocytes in the saline medium.

3.5. Test for naturally occurring antibodies

Before immunizations, all the selected donor-recipient pairs were tested for naturally occurring antibodies. Only 6 out of 85 samples were found to be positive for naturally occurring antibodies. The sera of these 85 buffaloes were tested with erythrocytes of 10 randomly selected buffaloes and 10 3+ve cattle cells. According to the reports available in the literature
naturally occurring antibodies are more frequently encountered during September to February. So, these buffaloes were bled in the month of February, in order to get the highest titre of the naturally occurring antibodies. No animal with naturally occurring antibody in its serum was included in the immunization panel for the production of isoimmune sera.

3.6. Production of polyvalent sera

For the production of reagents only isoimmunizations were done. Buffalo heifers from the general experimental herd were used for this purpose. The detailed schedule of immunizations is given in table 4. A total of 31 isoimmunizations were made, out of which 15 were single, 10 were reimmunizations and 6 were triple reimmunizations. The purpose of double and triple immunization was to get the desired titre of antibodies in the antisera. Apart from this, 3 cattle antisera were also produced to check the cross reaction of buffalo red blood cells with the cattle antisera.

On the suggestion of Dr. C.C. Cesterlee, who visited NDRI as an U.N.D.P. Expert on animal blood groups, a modified immunization method was adopted. This method was found advantageous over all the previous methods used for this purpose as it increases the antigenicity and reduces the period considerably during which a polyvalent sera is produced. Further, only very small quantity of packed red blood cells is needed for immunization. Thoroughly washed and well packed red blood cells were mixed with equal volume
of the adjuvant and then mixed well on a vortex mixer. Two ml of the antigen and adjuvant emulsion was injected in axilla muscles of the backjoint of the animal. After 15 days, the immunized animals were bled and the sera were tested for the potency of the antibodies produced. If the serum was found to be poor in titre value, the animal was reimmunized with the same dose of antigen. However, if the recipient did not respond to the antigenic doses, then the animal was given rest for one to four months and then reimmunized. This resulted in getting antisera of desired titre.

After ascertaining that the recipient has produced antibodies of high titre against its donor, the serum was harvested in bulk (approximately 1 litre). The antisera thus harvested were decomerimented and stored in small units in the deep freeze at -20°C till further use.

3.7. Fractionation of immune sera

The antisera obtained by immunizations are usually polyvalent. Fractionation of such antisera and the isolation of blood grouping reagents require thorough analysis by means of absorption and subsequent tests on the absorbed fractions. The detailed procedure for each reagent produced in these studies is given in the Chapter-4 and a general brief outline is given below.

Absorptions:— After ascertaining the titre of an antisera, all the cells which gave weak reactions were selected
for absorption. Properly diluted antisera at its highest titre was taken in glass test tubes. Different percentages of packed cells were tried for absorption. The tubes containing cells and antisera were kept at a temperature of 22-27°C for one hour and shaken gently after every ten minutes. These tubes were centrifuged for 10 minutes at 3000 rpm, and the supernatant was pipetted out and kept for subsequent testing with the panel cells including cells used for absorption.

In most of the antisera for making them monovalent, one, two, three and even four cells were used in combination for absorption when the single cell absorption did not produce a monovalent serum. The two cell absorption or even more than that was done subsequently, based upon the results of the lycic tests. Finally, before an antiserum was declared as monovalent blood group reagent, it had to pass the unity test. For this each serum was absorbed with all the positive cells in the panel and when the subsequent test showed that absorptions with each one positive cells removed antibodies for all other positive reacting cells, this was taken as a criterion for unity test and therefore the antisera can be used as a blood typing reagent.

Blood samples of 950 Murrah buffaloes, bulls and their progenies belonging to Punjab Agricultural University, Ludhiana and National Dairy Research Institute, Karnal, were taken for the present investigation. Blood samples (1-2 ml) were taken in sterilized test tubes, containing anticoagulant (Sodium citrate 2%, Sodium chloride 0.5%). The samples were collected at the farms and transported in icebox to the laboratories for storage and
and typing work.

3.6. Calculation of gene frequencies

Gene frequencies for each factor (based on dams only) were calculated assuming that each one of the antigen was controlled by two alleles. The presence of an antigen was indicated by the haemolytic test was taken to be dominant to its absence. Following formula was used to estimate the gene frequencies:

\[ q^2 = \frac{q}{N} \]

Where,

- \( q^2 \) = frequency of the recessive (non-reacting gene)
- \( q \) = Number of animals non-reacting.
- \( N \) = Total number of individuals tested.

3.9. Genetic studies

Associations among different blood group factors were worked out by Chi-square values obtained by 2 x 2 contingency tables using the following formula:

\[ x^2 = \frac{n(ad - bc) - n/2}{(a+b)(c+d)(a+c)(b+d)} \]
Blood factors

<table>
<thead>
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<tr>
<td>b</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>c</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>d</td>
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and \( n \) = total number of observations.

On the basis of these association values blood factors were divided into two groups and these groups were tentatively allotted to two loci. Inheritance of different phenogroups at these loci was studied. Number of alleles segregating at each loci were also found out.

3.10. Resolving disputed parentage cases

For making parentage studies, family data from the herds at National Dairy Research Institute and Punjab Agricultural University were used.

After careful examination of the blood group pictures of the sires, dams and their progenies, the cases of wrong parentage were collected based on the basic principle that any antigen possessed by the individual must be present in either or both of its parents. To resolve these doubtful cases, the pedigree records were checked in order to confirm the paternity of the individuals.
The artificial insemination register was also used to check the insemination dates of the doubtful cases. The information from this register alone resolved some cases of the wrong parentage, as shown in Table 6.