4. MATERIAL AND METHODS

4.1 METHODOLOGY:

The present study to determine Antigen frequencies of major blood group systems other than ABO was carried out on a total of 175 “O” Rh group samples of voluntary blood donors.

Out of these ‘175’ O Rh group samples, the antigen typing of ‘115’ samples was done by conventional Tube Technique and ‘60’ samples were antigen typed by column agglutination technique using Gel cards from Diamed, Switzerland. Performance of Conventional Tube Technique was compared with that of Column agglutination, as regards to ease in performing the assay, subjectivity in interpreting the assay and stability of the results. A comparison of cost involved in conventional tube technology and that of column agglutination technique was also made. After typing these 175 samples for antigens of Rh, Kell, Duffy, MNS, P, Lewis and Lutheran Blood Group systems, Red Cells from donors were selected for formulating inhouse screening and panel cells for Antibody detection and identification. Guidelines for the Transfusion services in the United Kingdom, 7th edition 2005\textsuperscript{162} and Code of federal regulations. Title 21 CFR Part 660.33 Washington, DC (Website : www.accessdata.fda.gov)\textsuperscript{163} were used while selecting red cells for formulating inhouse screening and panel red cells.

The performance of inhouse formulated screening and panel cells was then compared against the commercial red cells from Diamed, Switzerland & Immucor USA. For this purpose 100 blood samples from multitransfused patients were analysed for antibody detection and identification. These samples included 40 patients having chronic renal failure, 40 Thalassemia major patients and 20 multiparous females with Bad Obstetric History. All of these patients had a minimum of six transfusions.

This study was carried out at Blood Bank, Department of Pathology, Government Medical College, Surat from July 2000 to September 2013. The present study was duly approved by Human Research Ethics Committee of Government Medical College, Surat.
Sample size and Samples for the study:

Sample size for the present study to determine antigen frequency of major blood group system was obtained by using the sample size table freely available on the website www.research-advisors.com/tool/samplesize.htm.164

Table 68 Sample size requirement guideline.164

<table>
<thead>
<tr>
<th>Population Size</th>
<th>Confidence = 95%</th>
<th>Confidence = 99%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Margin of Error</td>
<td>Margin of Error</td>
</tr>
<tr>
<td>10</td>
<td>5.0%</td>
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<td>20</td>
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<td>600</td>
<td>234</td>
<td>340</td>
</tr>
<tr>
<td>700</td>
<td>248</td>
<td>370</td>
</tr>
<tr>
<td>800</td>
<td>260</td>
<td>396</td>
</tr>
<tr>
<td>1,000</td>
<td>278</td>
<td>400</td>
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<td>291</td>
<td>474</td>
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<tr>
<td>1,500</td>
<td>306</td>
<td>515</td>
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<tr>
<td>2,000</td>
<td>322</td>
<td>563</td>
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<tr>
<td>2,500</td>
<td>333</td>
<td>597</td>
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<td>3,500</td>
<td>346</td>
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<td>357</td>
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<tr>
<td>7,500</td>
<td>365</td>
<td>710</td>
</tr>
<tr>
<td>10,000</td>
<td>370</td>
<td>727</td>
</tr>
<tr>
<td>25,000</td>
<td>378</td>
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<td>381</td>
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<td>75,000</td>
<td>382</td>
<td>776</td>
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<td>100,000</td>
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<tr>
<td>100,000,000</td>
<td>384</td>
<td>784</td>
</tr>
<tr>
<td>300,000,000</td>
<td>384</td>
<td>784</td>
</tr>
</tbody>
</table>
Using this table, a sample size of 384 was thought to be appropriate for a population size ranging from 2,500,000 to 10,000,000 sufficing for a confidence level of 95% with a margin of error of 5%. However, due to the cost involved and financial constraints the actual sample size in the present study was reduced to 175 samples.

4 ml of blood samples in EDTA tubes were collected from “O” blood group regular and repeat voluntary donors after taking their consent for using their blood samples in the present study. Code of Federal regulations Title 21, CFR part 640.3 guidelines were used for meeting the criteria of donor suitability for preparing Reagent Red Blood Cells. For collecting the blood from these donors, guidelines 640.4 under Code of Federal regulations Title 21 were used (website www.accessdata.fda.gov). Tubes containing EDTA as anticoagulant were chosen for antigen typing as EDTA samples up to 14 days old are suitable for typing and they were already collected by the blood banks during the blood donation procedure as pilot samples. The samples were transported to the testing site at 2-6°C in coolant boxes. All the samples were processed for antigen typing within 48 hrs of collection.

These samples were collected from a) Voluntary blood bank, Sardar Smarak Hospital, Bardoli, b) Loksamarpan Raktadan Kendra, Varachha Road, Surat and c) Blood Bank, New Civil Hospital, Surat. The logic to collect blood samples from different blood banks was to have representative samples in the study from Urban, Semirural and Rural areas for South Gujarat, India.
**Sample Inclusion Criteria:**

- All the donor samples in the current study for antigen typing were selected only after confirming that their Direct Antiglobulin Test (DAT) results were negative because if DAT is positive due to IgG coating the cells, typing reagents employing the Indirect Antiglobulin Test (IAT) may give invalid results.\(^{168}\)

- All the samples were from donors, who had not been pregnant in last one year (in case of females) and who had not been transfused in last six months. This is because determination of Red cell phenotype in an individual can be difficult if the individual has been transfused, generally within 3 months.\(^{169}\) Phenotyping results on post transfusion samples can be misleading and should be interpreted with caution.

- As screening and panel cells were to be selected for antibody detection & identification in recipients from these donors and were to be used in future also, the donor chosen for the antigen typing were typically of age less than 30 yrs. All these donors had their contact details offered to the Blood Bank, Department of Pathology, Government Medical College, Surat so that they remain traceable and can be contacted as and when needed.

- EDTA samples showing partial or complete hemolysis were rejected and only clean unhemolysed samples were included in the study.

**Reagent and Equipments Used:**

Antigen typing of 175 “O” blood group donor samples was done for following blood groups: Rh (D, C, E, c, e), Kell (K, k, Kp\(^a\), Kp\(^b\)), Duffy (Fy\(^a\), Fy\(^b\)), Kidd (Jk\(^a\), Jk\(^b\)), Lewis (Le\(^a\), Le\(^b\)), P (P\(_1\)), MNS (M, N, S, s) and Lutheran (Lu\(^a\), Lu\(^b\)).

Out of these 175 samples, 115 random samples were processed for antigen typing by conventional tube technique using antisera following manufacturer’s instructions in the initial part of the study. Agglutination reaction in positive test results were recorded using Agglutination viewer and were graded as 1+ to 4+ as per the following table.
Table 69 Reading and Grading of serologic Reactions.\textsuperscript{170}

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description of Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>4+</td>
<td>One solid clump, no free cells, clear supernatant</td>
</tr>
<tr>
<td>3+</td>
<td>Several large clumps, clear supernatant</td>
</tr>
<tr>
<td>2+</td>
<td>Many medium-sized clumps, clear supernatant</td>
</tr>
<tr>
<td>1+</td>
<td>Numerous small clumps, cloudy red supernatant</td>
</tr>
<tr>
<td>±</td>
<td>Numerous very small clumps easily dispersed, cloudy red supernatant</td>
</tr>
<tr>
<td>(+)</td>
<td>Appears negative macroscopically, but agglutination visible microscopically</td>
</tr>
<tr>
<td>0</td>
<td>Negative, no agglutination, seen macroscopically or microscopically</td>
</tr>
<tr>
<td>H</td>
<td>Complete hemolysis, no intact RBCs remaining</td>
</tr>
<tr>
<td>PH</td>
<td>Partial hemolysis, some RBCs still intact, but hemolysis visible in supernatant</td>
</tr>
<tr>
<td>mf</td>
<td>Mixed field- mixture of agglutinated and unagglutinated cells</td>
</tr>
</tbody>
</table>

The antisera used for the study were from Diamed, Switzerland & Immucor, Gamana, USA. Each of these 115 samples was antigen typed with two sources of antiserum (Diamed as well as Immucor). This was in accordance with the guidelines for the Blood Transfusion Services in the United Kingdom, 7\textsuperscript{th} Edition 2005, which states that when testing reagent red cells, in order to confirm the presence of absence of antigens listed in the antigen profile, a sample from each individual should be tested whenever possible, with a minimum of two antisera for each specificity prepared from different donors / cell lines.\textsuperscript{171}

In these 115 samples typing for D, C, c, E, e, K, Jk\textsuperscript{a}, Le\textsuperscript{a}, Le\textsuperscript{b}, P, P1, N antigen was done using monoclonal antisera while K, Kp\textsuperscript{a}, Kp\textsuperscript{b}, Fy\textsuperscript{a}, Fy\textsuperscript{b}, Jk\textsuperscript{b}, S, s, Lu\textsuperscript{a}, Lu\textsuperscript{b} antigens were typed using polyclonal antisera employing indirect Antiglobulin Test Method. The Polyspecific Antihuman Globulin reagent used in the study was from Diamed, Switzerland.
4.2 Procedure employed for phenotyping Red Blood Cells with Agglutinating antibodies (Monoclonal Antibodies):

Preparation of Blood Sample:

A 3-5% of red cell suspension in isotonic saline solution of the red cells to be phenotyped was prepared as following:

1) 1 ml of Isotonic saline was dispensed in a clean glass tube.
2) 2 drops (100 μl) of whole blood was added to the isotonic saline and mixed gently.

Controls:

Cells for positive and negative controls were chosen from the commercial red cell panels of Diamed & Immucor. For Negative Control, RBCs lacking the antigen corresponding to the specificity of the antiserum in use were selected. For positive control RBCs known to carry the antigen in single dose corresponding to the specificity of the antiserum in use were selected. For eg. when testing anti-M and/or anti-N, M+N+ RBCS were selected.

Test procedure:

1) All the monoclonal antisera were allowed to reach from temperature (18-25\(^{0}\)C) before use.
2) A clean glass tube (12 x 75 mm size) was appropriately labeled.
3) 1 drop (50 μl) of the monoclonal antiserum was dispensed in to the appropriate tube.
4) 1 drop (50 μl) of red cell suspension was added into the appropriately labeled tube.
5) Mixed well and incubated for 5 minutes at room temperature (18-25\(^{0}\)C).
6) Tube was centrifuged for 20 seconds at 1000 g.
7) Cells were resuspended gently after centrifugation and examined macroscopically for agglutination.
Results:

An agglutination of >2+ was confirmed in single dose control RBCs before interpreting the test results.

**Positive**: Agglutination of + to ++++ indicated of a reaction between the antibody and the red cells.

**Negative**: No visible agglutination indicated that no reaction has taken place between the antibody and the red cells.
Procedure employed for phenotyping Red Blood Cells with IAT-Reactive (Polyclonal) antibodies:175,176

Preparation of Blood Samples:
A 3-5% of red cell suspension in isotonic saline solution of the red cells to be phenotyped was prepared as follows:
1) 1 ml of isotonic saline was dispensed in a clean glass tube.
2) 2 drops (100 μl) of whole blood was added to the isotonic saline and mixed gently.

Test Procedure:
1) A clean glass tube (12 x 75 mm) was appropriately labeled.
2) 1 drop (50 μl) of the blood typing serum of desired specificity was dispensed into the appropriate tube.
3) 1 drop (50 μl) of red cell suspension was added into this labeled tube.
4) The contents of the tube were mixed well and incubated at 37°C in a water bath for 30 minutes.
5) The contents of the tube were washed with isotonic saline for 3 times and the supernatant was carefully removed.
6) 2 drops of Coombs serum was then added and all the contents were mixed well.
7) The tube was then centrifuged for 1 minute at 1000 rpm.
8) After centrifugation, the cells were resuspended gently and then observed macroscopically for agglutination.
9) All the negative results were confirmed by adding coombs control cells (check cells).

Controls:
Cells for positive and negative controls were chosen from the commercial red cell panels of Diamed & Immucor. For negative control, RBCs lacking the antigen corresponding to the specificity of the antiserum in use were selected. For positive control, RBCs known to carry the antigen in single dose corresponding to the specificity of the antiserum in use were selected. For eg. When testing with anti-S and/or anti-s, S+s+ RBCs were selected as positive control.177
Results:
Reactions by IAT with single-dose antigen-positive RBCs ≥ 2+ were confirmed before interpreting the test results.

Positive: Agglutination of + to ++++ indicated a reaction between the antibody and the red cells.

Negative: No visible agglutination indicated that no reaction has taken place between the antibody and the red cells.

The Coomb’s control cells (check cells) used in the study were a combination of inhouse prepared cells and ready to use cells from Diamed, Switzerland. For preparing in house Coomb’s control cells, Anti-D (IgG) from Tulip Diagnostics (P) Ltd. India & Diamed, Switzerland were utilized.

Procedure used for preparing Coombs Control Cells (check cells):[178,179]

1) Freshly collected O Rh (D) positive red blood cells were washed with isotonic saline for three times.

2) After the third wash, the supernatant was decanted thoroughly. To the cell button 5 ml of Anti-D (IgG) reagent was added and the red blood cells were resuspended.

3) The mixture was incubated at 37°C for 15 minutes.

4) After incubation, the sensitized red blood cells were thoroughly washed with isotonic saline for four times.

5) After the last wash, the supernatant was thoroughly decanted.

6) From the cell button, a 3% red cell suspension was prepared for further use as Coombs control cells and stored at 2-8°C.

Validation of prepared 5% Coombs control suspension:
1) 1 drop of Coombs control cells were dispensed into a test tube.

2) Two drops of Anti human reagent were added to this cells.

3) The mixture was centrifuged for 1 minute at 1000 rpm.

4) After centrifugation, the cell button was very gently resuspended and observed macroscopically for agglutination.
The potency of the commercial antisera used for phenotyping of donor red cells for major blood group systems was verified using the following criteria:\textsuperscript{180,181}

Blood grouping Reagents recommended for the test tube methods, including the indirect antiglobulin tests shall have the following potency titer values.

1) For Anti-K, Anti-k, Anti-Jka, Anti-Fya at least 1+ reaction with a 1:8 dilution of the reagent.
2) For Anti-S, Anti-s, Anti-P1, Anti-M, Anti-I, Anti-e (Saline), Anti-c at least 1+ reaction with a 1:4 dilution of the reagent.

Appropriate red cells (Single dose cells) were chosen from commercial panels of Diamed, Switzerland & Immucor Gamma USA, for confirming the potency of commercial antisera before using them in antigen phenotyping of donor red cells.

In the later part of the study, 60 ‘O’ blood group donor samples were processed for antigen typing by dextran acrylamide gel technique. Four types of gel cards were used from Diamed, Switzerland. In these 60 ‘O’ blood group donor samples, selected cells were used for antigen typing. The reason for this selection process was that in the initial 115 blood samples, no sample turned out to be R2R2 and rr and enough number of Ror, rr, r’r’, r’r, r’r’’ or rr” were not obtained which could be used in panel cells. To get 5 R2R2 phenotypes, 600 ‘O’ positive samples were tested with antisera-e, anti-E, anti-C and anti-c.

First card used was Diaclon Rh-subgroups + K, for phenotyping C, c, E, e and K antigens. The second type of card used was ID-Antigen profile I for typing P1, Lea, Leb, Lu, Lu antigens. The third card used was ID-Antigen profile-II for phenotyping k, Kpa, Kpb, Jka, Jkb. The fourth card used was ID-card Antigen Profile-III for determining M, N, S, s, Fya, Fyb antigens.
4.3 Procedures employed for Phenotyping Red Cells by Gel Technique:

Procedure for using Diaclon Rh-Subgroups +K Card:

Preparation of Blood Samples:
A 5% red cell suspension in ID-Diluent 2 was prepared as follows:
ID-Diluent 2 procured from Diamed Switzerland was allowed to reach room temperature before use.

1) 0.5 ml of ID-Diluent 2 was dispensed into a clean test tube.
2) 50 μl of whole blood from the donor sample to be tested was added to it and mixed gently.
3) The formulated cell suspension was used immediately.

Test Procedure:
1) Before using the Diaclon – Rh-subgroups + K card, it was confirmed that there were no signs of drying, bubbles, damaged seals, drops of gel or supernatant in the upper part of the microtubes or on the underside of the aluminium foil.
2) The ID Card was labeled appropriately.
3) The aluminium foil from cad was removed by holding the ID card in the upright position and 10 μl of red cell suspension in ID-Diluent 2 was added to the microtubes in the card.
4) The ID- Card was centrifuged for 10 minutes in the ID-centrifuge.
5) The results were read & recorded.

Interpretation of the results:
Positive: Agglutinated cells forming a red cell line on the surface of the gel or agglutinates dispensed in the gel.

Negative: Compact button of cells on the bottom of the microtube.
A positive reaction (+ to ++++) indicates presence of the corresponding antigen.
Reactions of \( \leq 2^+ \) may indicate the presence of weak or variant forms of the antigen.
A negative reaction indicates absence of the corresponding antigen.

**Procedure employed for using ID-Antigen Profile I & II cards:**

**Preparation of Blood Sample:**
A 5% red cell suspension in ID-Diluent 1 was prepared as follow.
ID-Diluent 1 procured from Diamed, Switzerland was allowed to reach room temperature before use.

1) 0.5 ml of ID-Diluent 1 was dispensed in a clean test tube.
2) 50 \( \mu l \) of whole blood from the donor sample to be tested was added to it and mixed gently.
3) The tube with its content was incubated for 10 minutes at room temperature (18-25\(^{0}\)C).
4) The red cell suspension was then used within 15 minutes after incubation.

**Test Procedure:**
1) Before using the ID-Antigen profile I & II cards, it was confirmed that there were no signs of drying, bubbles, damaged seals, drops of gel or supernatant in the upper part of the microtubes or on the underside of the aluminium foil.
2) The ID card was labeled appropriately.
3) The aluminium foil from the card was removed by holding the ID card in the upright position and 10 \( \mu l \) of red cell suspension in ID-Diluent 1 was added to the microtubes in the card.
4) The ID-card was centrifuged for 10 minutes in the ID-centrifuge.
5) The result were read and recorded.

**Interpretation of the results:**

**Positive:** Agglutinated cells forming a red line on the surface of the gel or agglutinates dispersed in the gel.
**Material and Methods**

**Negative**: Compact button of cells on the bottom of the microtube.

Positive reactions of + to ++++ indicate presence of the corresponding antigen.

A double population must also be considered as positive. However, a double population may also indicate the presence of cells positive and negative for the corresponding antigen.

Negative reactions indicate absence of the corresponding antigen.

Strong ++++ reactions are very rare.

Anti Jk\(^a\) may react more strongly with Jk (a+b-) than with Jk (a+b+) red blood cells (dosage effect).

**Procedure employed for using ID-Antigen Profile-III Card**:

**Preparation of Blood Sample**:

A 0.8% red cell suspension in ID-Diluent 2 was prepared as follows:

ID-Diluent 2 procured from Diamed Switzerland was allowed to reach room temperature before use.

1) 1 ml of ID-Diluent 2 was dispensed into a clean test tube.

2) 20 \(\mu\)l of whole blood from the donor sample to be tested was added to it and mixed gently.

3) The formulated cell suspension was used immediately.

**Test Procedure**:

1) Before using the ID-Antigen Profile III card, it was confirmed that there was no signs of drying, bubbles, damaged seals, drops of gel or supernatant in the upper part of the microtubes or on the underside of the aluminium foil.

2) The ID card was labeled appropriately.
3) The aluminium foil from the card was removed by holding the ID card in the upright position and 50 μl of red cell suspension in ID-Diluent 2 was added to the microtubes in the card.

4) 50 μl of the ID-test sera (anti-M, -N, -S, -s, -Fya, -Fyb) separately provided was added to the appropriate microtube of the card.

5) The ID card was then incubated for 10 minutes at room temperature (18-25°C).

6) After incubation, the ID – card was centrifuged for 10 minutes in the ID-centrifuge.

7) The results were read and recoded.

**Interpretation of the results:**

**Positive:** Agglutinated cells forming a red line on the surface of the gel or agglutinates dispersed in the gel.

**Negative:** Compact button of cells on the bottom of the microtube.

Positive reactions of + to ++++ indicate presence of the corresponding antigen.

A double population must also be considered as positive. However, a double population may also indicate the presence of cells positive and negative for the corresponding antigen.

Negative reactions indicate absence of the corresponding antigen.

Strong ++++ reactions are very rare.

Anti Jk^a may react more strongly with Jk (a+b-) than with Jk (a+b+) red blood cells (dosage effect).

From data obtained by antigen typing of the 175 ‘O’ Rh group samples, appropriate cells were selected and screening and panel red cells were formulated in house. For this purpose, Guidelines for the Transfusion services in the United Kingdom, 7th Edition 2005, Code of Federal Regulations Title 21 code. 660.33 were referred.
Reagent red cells for use in antibody screening:

The detection of irregular antibodies in the serum of a patient is of greater clinical significance than if such antibodies are detected in blood donors. Reagent red cells of a lesser specification may be used when performing antibody screening tests on blood donor samples.

In general the following criteria were used.

- Reagent red cells for used in antibody screening were confirmed as group O.
- Where practicable, reagent red cells known to express antigens having a frequency of less than 1% in the general population were not included in reagent red cells for antibody screening.
- Where practicable, red cells from individuals known consistently to effect troublesome reactions with HLA antibodies were not used as reagent red cells for antibody screening.

Reagent red cells for use in antibody screening of patient samples:

- As a minimum the following antigens were expressed on the reagent red cells for antibody screening: C;c;D;d;E;e;K;k;Fy^a;Fy^b;Jk^a;Jk^b;S;s;M;N;P1;Le^a; Le^b; Lu^a and Lu^b.
- As a minimum, reagent red cells from two individuals were provided. One reagent red cells was R2R2; the other R1R1.
- Apparent homozygous expression of the following antigens was included; Fy^a;Fy^b;Jk^a;Jk^b;S and s.

Reagent red cells for use in antibody identification:

- The antigen profile of reagent red cells for antibody identification permits the identification of frequently encountered antibodies, for example anti-D, anti-E, anti-K, anti-Fy^a, and of commonly encountered alloantibody mixtures, for example, anti-D+K.
- Red cell antibody identification comprised of red cells from 11 individuals expressing between them the following antigens:
  C;c;D;d;E;e;K;k;Fy^a;Fy^b;Jk^a;Jk^b;S;s;M;N;P1;Le^a; Le^b; Lu^a and Lu^b.
- Red cells from one individual was R1R1 and from another R1R1 were selected and between them were expressing the following antigens: K;k;Fy^a;Fy^b;Jk^a;Jk^b;S and s.

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Material and Methods

- Red cells from one individual was R₂R₂, another from r’r and one another from r’r were chosen for formulating antibody identification panel cells.
- Red cells from a minimum of three individuals lacking the Rh antigens C,E and D. One of these three individuals was K positive. Between them, red cells from these individuals were exhibiting apparent homozygous expression of the following antigens: c;k; Fyᵃ;Fyᵇ;Jkᵃ;Jkᵇ;S and s.

4.4 Inhouse formulated Screening and Panel Red Cells.

### Inhouse formulated screening cells.

<table>
<thead>
<tr>
<th>Vial</th>
<th>Donor</th>
<th>Rh-Hr</th>
<th>Kell</th>
<th>Duffy</th>
<th>Kidd</th>
<th>Lewis</th>
<th>P</th>
<th>MN</th>
<th>Lutheran</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>R₁R₁</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
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<tr>
<td>II</td>
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<td>+</td>
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<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

The screening and panel cells were stored in Alsever’s solution, because the Alsever’s solution contains the necessary nutrients for in-vitro storage of RBCs for reagent use. This formulation includes antibiotics to retard bacterial growth. RBCs stored in Alsever’s solution at 4°C maintain their viability for several weeks.¹⁸⁵
Reagents:

1. Chloramphenicol: 0.33 g
2. Citric acid – Monohydrate (C₆H₉O₇.H₂O): 0.55 g
3. Dextrose (C₆H₁₂O₆): 20.5 g
4. Inosine: 2.0 g
5. Neomycin sulphate: 0.5 g
6. Sodium Chloride (NaCl): 4.2 g
7. Trisodium Citrate (C₆H₅Na₃O₇.2H₂O): 8.0 g

Procedure to formulate Alsever’s solution:

1. Dissolve citric acid, dextrose, sodium chloride and trisodium citrate in approximately 600 ml of distilled water.
2. Add chloramphenicol, inosine and neomycin sulphate; mix well.
3. Dilute to 1 L with distilled water.
4. Store at 4°C.

Screening and panel red cells were suspended on Alsever’s solution at a concentration of 3% and stored at 2-6°C. The vials containing the screening and panel cells were labeled appropriately and date of manufacture of the cells was the date of blood withdrawn from the donor. This was in accordance with the guidelines of Code of Federal Regulations Title 21, Sec. 660.34.¹⁸⁶
4.5 Validation of inhouse screening and panel Red cells :

For Validation In house Screening and Panel Red Cells, three parameters were chosen 1) Limit of detection, 2) Shelf life, 3) Performance evaluation.\textsuperscript{187}

1) Limit of detection :

For the purpose of limit of detection, Antisera, Anti-Fy\textsuperscript{a} and Anti-D from Diamed were diluted up to 32. This particular diluted antisera were tested with inhouse screening and panel cells for antibody detection and identification. Screening cells were able to detect and panel cells were able to identify both the diluted antibodies.

2) Shelf Life :

To define the shelf life of the inhouse screening and panel cells, commercial Anti Fy\textsuperscript{a} and Anti-D antisera were diluted up to 32 and were tested every week till day 56 (8 weeks) by considering the first day of test as zero. The inhouse screening and panel cells were able to detect and identify these diluted antibodies.

Moreover the stability of screening and panel cells was evaluated throughout the shelf life of 8 weeks by checking the presence of Fy\textsuperscript{a}, Fy\textsuperscript{b}, M & P\textsubscript{1} antigens which are prone to deteriorate on storage using commercial antisera of desired specificity.\textsuperscript{188}

3) Performance evaluation :

For this purpose, a total of 100 blood samples in plain tubes were selected from multitransfused patients reaching the blood banks for transfusion requirement. The volume of the sample collected was 15 ml which could suffice for detection and identification purpose. These 100 samples included 40 patients having chronic renal failure, 40 having Thalassemia major and 20 were Multiparous females with Bad Obstetric History. All of these patients had minimum of six transfusions.
All of these 100 samples were reacted with inhouse screening cells formulated in the present study and also with screening cells from Diamed Switzerland and Immucor Gamma, USA. The methodology used was as per the kit literature of Diamed, Switzerland & Immucor, USA.

The samples which revealed the presence of antibody were further processed for antibody detection using inhouse panel red cells, and panel red cells from Diamed, Switzerland & Immucor Gamma, USA.

Results obtained were then statistically evaluated.