Symposium on Fest-Schrift for Late Dr. P.M. Udani

Thalassemia and Related Hemoglobinopathies

Sharada A. Sarnaik

Carman and Ann Adams Department of Pediatrics, Wayne State University School of Medicine, Pediatric Hematologist/Oncologist, Director, Sickle Cell Center, Children's Hospital of Michigan, Detroit, MI, USA.

Abstract. Hemoglobinopathies are the most common single gene disorders in man. There are several hundred of these disorders though the thalassemias - alpha and beta and the sickling disorders make up the vast majority. Recent advances in the understanding of the hemoglobin structure and the genetics of its synthesis has contributed significantly to the understanding of these diseases. Disorders include those with reduced globin synthesis, abnormal globin chains and failure to switch globin chain synthesis at the appropriate age. This review focuses on the clinical features, diagnosis and management strategies of the alpha and beta thalassemias, the sickling disorders and touches on a few rarer hemoglobinopathies. It also emphasizes prevention strategies and chronic transfusion safety in countries like India where there are limited resources.
[Indian J Pediatr 2005; 72 (4) : 319-324] *E-mail : ssarnaik@med.wayne.edu.*

Key words : *Hemoglobinopathies; Thalassemias; Sickle cell disease; Management; Prevention*

The inherited hemoglobin disorders are the most common single gene defect in man. The frequency of the carrier state has been estimated to be 270/million with about 400,000 annual births a year of infants with serious hemoglobinopathies. The prevalence of hemoglobinopathies is on the rise worldwide. This is of special importance in developing countries, where it increases the burden of health care delivery systems.

NORMAL HEMOGLOBIN STRUCTURE

Hemoglobin is a tetrameric protein with four peptide chains, two α *or* α*-like chains* and two non- α (*or* β*-like*) chains. The molecule is held together by interactions between peptide chains. The amino acids in the globin polypeptide chain are assembled in a long convoluted knot within which lies the heme moiety, which carries a molecule of O_2. Its iron is in the Fe^{++} form, and does not change its valence with release of O_2. Abnormalities that result in a change to the ferric (Fe^{+++}) form, results in a hemoglobin molecule that is unable to carry O_2. (methemoglobin, hemoglobin M). The α1/β2 interface is an important region for the unique O_2-carrying function of hemoglobin, and abnormalities in this area will alter its O_2 affinity. The areas of the molecule where the globin chains are in contact with each other or with the heme molecules are functionally important and have been highly conserved throughout evolution.

GENETICS OF HEMOGLOBIN SYNTHESIS

Hemoglobin synthesis is directed by controlling genes which are switched on and off at certain stages of human life, resulting in different globin chain synthesis at different ages. The genes controlling α-like globin chains are located on chromosome 16, while genes for β-like chains are located on chromosome 11. Globin gene expression is under intense research scrutiny because of therapeutic possibilities of preferentially stimulating hemoglobin F synthesis (by gene manipulation) to ameliorate β chain disorders. Switch from γ chain to β chain production starts at about the 9th gestational week, when hemoglobin A becomes detectable. Fetal hemoglobin synthesis declines but persists until 9 months of age, at which time the switch is complete. (*This also accounts for the fact that* β *chain abnormalities do not manifest at birth).* A small amount of fetal hemoglobin (1% or less) persists in adults in a small clone of cells called F cells. A third minor normal hemoglobin seen in adults is Hemoglobin $A_2(\alpha_2/\delta_2)$. Normal A_2 levels are 1 to 3%. The importance of Hemoglobin A_2 is that *it is diagnostically elevated in the beta thalassemias.* To summarize, normal hemoglobins are Hemoglobin A= 96%, Hemoglobin F= 1%, Hemoglobin A_2=3%.

CLASSIFICATION OF ABNORMAL HEMOGLOBIN SYNTHESIS

1. Production of structurally normal, but decreased amounts of globin chains (the thalassemias)
2. Production of structurally abnormal globin chains; (e.g. Hemoglobin S, Hemoglobin C, Hemoglobin E)
3. Failure to switch globin chain synthesis (hereditary persistence of fetal Hemoglobin, a condition that influences severity of clinical

Correspondence and Reprint requests : Dr. Sharada A. Sarnaik, Children's Hospital of Michigan, 3901 Beaubien, Detroit, MI 48201. Fax : 313-745-5237.

manifestations of other hemoglobinopathies).

Inheritance of all of the above disorders is autosomal codominant. "Codominant" is the most accurate terminology, because being heterozygous carries discernible but minor clinical findings.

THALASSEMIAS

Definition

This is a genetic decrease in globin chain synthesis. Theoretically, there are as many types of thalassemias as there are types of globin chains. The most clinically relevant are the α and the β-thalassemia.

Genetics

The thalassemias are all caused by mutations in the globin gene cluster. The defects are numerous, (more than 100 different mutations have been described), and include deletional or non-deletional mutations. Mutations usually have a geographic and ethnic distribution.

α-Thalassemias

Definitions & Clinical Features

α-thalassemias are characterized by decreased α chain synthesis. The most common defect is deletional, although non-deletional defects have been described. The α gene is duplicated, and there are 2 α globin genes per haploid genome; thus the abnormality can result from one to four gene deletions. Recently accepted classification of the α-thalassemias is similar to the β thalassemias; that is, α^0 where no normal α globin is produced by the gene, and α^+ where there is reduced globin product. Where there is a single gene deletion and three intact genes there is no discernible abnormality, and this is called the silent carrier state. The two gene deletion is a minor clinical condition with a mild hypochromic, microcytic anemia, similar to iron deficiency. Deletion of three genes, or Hemoglobin H disease results in moderate anemia that is hypochromic and microcytic; there is hepato-splenomegaly due to extra-medullary hematopoeisis. Deletion of all four genes is incompatible with life and results in hydrops fetalis or intra uterine death (Table 1).

Diagnosis

Routine CBC shows hypochromia, microcytosis and mild anemia in the 2 and 3 gene deletions. Confirmation of decreased α chains is done by globin chain synthesis measured in reticulocytes (expensive and difficult to do). Restriction fragment polymorphism is reserved for prenatal diagnosis. Decreased a chain results in an excess of non-α chains, which are insoluble and form tetramers. These abnormal hemoglobin tetramers can be demonstrated by the presence of red cell inclusions on cresyl blue stain and by hemoglobin electrophoresis. They are rapid moving and faster than hemoglobin A requiring special attention during electrophoresis testing. Bart's hemoglobin (γ-4 tetramers) is found in the first few weeks of life and hemoglobin H (β-4 tetramers) can be found in older patients.

Since the genes for α and β-thalassemia are on separate chromosomes, they can be co-inherited. Co-inheritance of α thalassemia can have beneficial effects on the phenotype (clinical severity) of β thalassemia as well as the structural hemoglobinpathies, for example sickle cell disease.

β-Thalassemia

Inheritance of one gene for β thalassemia results in thalassemia trait (also called thalassemia minor). This condition can be diagnosed by simple screening for microcytosis and a relatively high red cell count. Population screening has been effectively carried out in India by using the Naked Eye Single Tube Redcell Osmotic Fragility (NESTROFF) technique. NESTROFF has been compared to hemoglobin electrophoresis by HPLC (high performance liquid chromatography) by several authors and while not highly specific, it has been found to be highly sensitive (very few false negatives).[1,2] It is relatively inexpensive and simple and thus suited for large-scale population screening for thalassemia trait. Genetic counseling for couples at risk for offspring with homozygous β-thalassemia may be done using NESTROFF.

Pathophysiology and Clinical Features of Thalassemia Major

The decreased β chain synthesis in this condition results in an excess of α chains, some of which are used for synthesis of other hemoglobins which do not have β chains, such as hemoglobin F (α2 γ2) or hemoglobin A_2 (α2 δ2), which are then elevated. Free α chains left over form tetramers, which are very insoluble. They accumulate and precipitate within the RBC, leading to increased fragility and cell death. The RBC life span is thus very short and they may be destroyed within the marrow leading to ineffective erythropoeisis. The lack of

Table 1. Detection of Genes

Phenotype	# α genes	α/β synthetic ratio	Haplotype	Genotype
Normal	4	1.0	α, α	α α/ α α
Silent carrier	3	0.8	α^+, α	- α / α α
2 gene deletion-α thal 1	2	0.6	α^0, α or α^+, α^+	—/ α α or - α /- α
Hemoglobin H disease	1	0.3	α^0, α^+	—/- α
Hydrops fetalis	0	0	α^0 , α^0	—/—

β chains leads to decreased hemoglobin content per cell, hypochromia and microcytosis. Attempts to increase the red cell mass result in expanded marrow cavities and extramedullary erythropoeisis in the liver and spleen. Children with thalassemia major present at about 6 months of age with anemia that can be severe and symptomatic. Growth failure, cardiac dysfunction, pallor, jaundice, and hepatosplenomegaly are commonly seen. They become iron-loaded even with sparingly used transfusions because of increased iron absorption from the diet. Iron toxicity affects the liver (cirrhosis), pituitary (hypogonadism and growth failure), heart (arrhythmia and cardiomyopathy) and bone (pathologic fractures).

Management Strategies

1. Regular blood transfusions are used to avoid growth failure and other pathologic consequences of severe anemia. 2. Splenectomy is usually performed if there is increased transfusion requirement from "hypersplenism" 3. Iron chelation therapy with deferioxamine by 12 hourly subcutaneous infusions on a daily basis via battery-operated pumps is essential to avoid iron overload. 4. Folic acid supplementation is needed because of increased requirements. 5. Bone marrow transplantation where chronic transfusions and chelation are not possible.

Chelation Strategies

The alternative to parenteral deferioxamine is the oral chelator, -deferipone. Significant dose-related side effects are neutropenia, arthropathy and agranulocytosis. There have been conflicting reports of increased hepatic fibrosis as well.[3-5] It has the merits of being effective, relatively inexpensive and much simpler to administer.

STRUCTURAL HEMOGLOBINOPATHIES

These defects usually result from a point mutation in the α or β globin gene, which produces an erroneous amino acid insertion in the polypeptide chain of the hemoglobin molecule. The effect is a functional abnormality whose effects depend on where the mis-sense mutation occurs. The mutation could result in: (a) No physiological abnormality and no clinical problem, (b) An increased tendency to aggregate (HbS, HbC), (c) Instability of the hemoglobin molecule resulting in a hemolytic anemia, (d) Altered O_2 affinity (increased or decreased), (e) Decreased O_2 carrying capacity (Hb M). There are more than 400 different abnormal hemoglobins described to date. Fortunately, most are rare and cause no clinical disease. Sickle cell disease is a frequent and clinically relevant hemoglobinopathy.

Sickling Disorders

1. SS - homozygosity for the mutant Hb S. (No normal β alleles, no HbA, also called sickle cell anemia)
2. SC - double heterozygosity for two β chain mutants, Hb S and Hb C, (No normal β alleles, no Hb A).
3. Sickle-β thalassemia - double heterozygosity for Hb S and β-thalassemia. One β gene directs the synthesis of Hb S, the other is either completely suppressed and the patient has no Hb A, (S β^0 thal) or incompletely suppressed and the patient produces a small amount of Hb A (S β^+ thal). Hb A_2 is elevated in these conditions.
4. SO Arab and SD - Double heterozygosity for Hb S and Hb O or D, respectively.
5. Hereditary persistence of fetal Hb with Hb S, where there is a failure to switch from γ to α β chain synthesis co-inherited with Hb S. Patients have high levels of HbF, and have very mild clinical symptoms because of its protective effects.

Pathophysiology of Sickling

In its deoxygenated state, hemoglobin S is extremely insoluble. Polymer formation within the RBC causes a shape change to the sickled form that gives the disease its name. Sickling is accompanied by increased rigidity, loss of deformability, increased adhesiveness to endothelial cells and red cell membrane damage, all of which adversely affect the flow properties of the red cells through the microvasculature. This produces vaso-occlusion, which sets up a vicious cycle of stasis, low PO_2 in tissues, tissue acidosis, increased viscosity, further polymer formation and more vaso-occlusion to complete and perpetuate the cycle. Membrane damage causes the red cell life span to be short (15 days instead of 120 days), resulting in a hemolytic anemia.

Clinical Features

All the sickling disorders mentioned above are associated with similar clinical features; the double heterozygous states generally have a milder clinical course.

1. Hemolytic anemia results in pallor, jaundice, increased fatigue, gallstones, and poor growth.
2. Aplastic crises occur following viral infections, where there is transient marrow suppression resulting in a life-threatening fall in Hb level.
3. Vascular obstruction from intravascular sickling results in episodic musculoskeletal pain, which is variable, unpredictable and can be disabling if very frequent and severe. Although called "pain crises", pain attacks are usually uncomplicated and not life threatening. Specific areas where typical obstruction occurs are (a) Acute bone pain or infarction is very common at all ages. (b) Dactylitis, -a swelling of the hands and feet, is a classic early childhood symptom. (c) Osteonecrosis of the spine and femoral heads is often seen in adults, and commonly causes chronic pain. (d) Splenic sequestration crisis - a sudden pooling of blood in the spleen with hypovolemic shock, a life-threatening and recurrent syndrome of early childhood. (e) Strokes - Uncommon, but commonly recurrent and a problem with high morbidity.

Ischemic and haemorrhagic catastrophies can occur. (f) Acute chest syndrome - pulmonary infarction and/or pneumonia, is common, and indistinguishable from each other. (g) Beginning in early childhood there is a lifelong risk of severe bacterial infections, which are often fatal. This is due to a loss of splenic function (auto-splenectomy) from vaso-occlusion and fibrosis. (h) Renal manifestations – there is often a loss of urine concentrating capacity due to sickling in vessels around the loop of Henle; large volumes of dilute urine are produced even in young children, underscoring the need for copious fluid intake at all times to avoid dehydration. Other renal problems include hematuria and glomerular nephropathy. i) Other manifestations of vaso-occlusion include priapism, trophic leg ulcers, and blindness.

The disease is extremely variable in its severity. Factors, which affect disease severity, have not been clearly defined, and are the subject of research. These include (1) the presence of genetic markers such as the β gene haplotype, (2) the co-inheritance of α-thalassemia (beneficial) and (3) the amount and distribution of Hb F (higher levels are beneficial).

Sickle Cell Trait

This is a benign, carrier state, and the vast majority of individuals have no clinical symptoms. The incidence of the trait in U.S. blacks is 10%. In certain tribal areas of India the incidence approaches 30 %. Sickle cell trait can cause hematuria and a loss of urine concentrating capacity. Symptoms from intravascular sickling have been reported with strenuous exercise at high altitudes and flying at high altitudes in un-pressurized aircraft.

Diagnosis

1. The patient's clinical history and physical findings;
2. The presence of Hb S shown by the inexpensive and highly sensitive solubility test even in the carrier state. Hemoglobin electrophoresis confirms the exact phenotype.
3. The presence of hemolytic anemia (low hemoglobin, high reticulocyte counts, bilirubin, and LDH) and morphologic sickling on blood smears.

Management Strategies

1. Symptomatic and supportive care of complications such as pain episodes with: analgesics, (often narcotics), local heat packs, adequate hydration, acid-base balance, avoiding hypoxia and exposure to cold, and treatment of febrile episodes early and aggressively with antibiotics.
2. Judicious use of blood transfusions - to prevent strokes in children, to treat pneumonia if there is respiratory distress, for severe anemia (Hb < 5, usually with aplastic crisis or splenic sequestration), or for problem pregnancies.
3. Early diagnosis by screening of newborns, and routine daily prophylactic penicillin and pneumococcal vaccine are used to prevent the high childhood mortality from infections.
4. Psychosocial support and self-help groups are important for improved disease adjustment, especially for adults. Pain attacks are often capricious, severe and frequent and can be an important barrier to self-determination and independent living.
5. Recent advances have brought research strategies to the bedside: *(a)* Agents which stimulate fetal hemoglobin production such as hydroxyurea, *(b)* Bone marrow or cord blood stem cell transplantation from an HLA identical sibling is a high-risk procedure which can be curative. It is used for certain individuals with markers for adverse outcome, such as stroke in a young child. *(c)* Anti-sickling agents such as membrane-active drugs, and *(d)* Gene therapy is an approach of the future.

RARER STRUCTURAL HEMOGLOBINOPATHIES

1. The Unstable Hemoglobins – These usually occur from aminoacid substitutions near the heme pocket resulting in a Hb which is unstable. A prototype is hemoglobin Zurich. The instability causes a tendency of the heme to separate from the globin chain with the slightest oxidative stress. The denatured hemoglobin precipitates in the red cell and forms Heinz bodies, which cause the cells to sequester in the spleen resulting in a hemolytic anemia.

Diagnosis is by demonstration of a hemolytic anemia, detection of Heinz bodies (by staining), and the heat precipitation test. Hemoglobin electrophoresis is not always useful because of the tendency of the hemoglobin to rapidly denature.

One should avoid oxidant drugs, transfuse as clinically indicated, and splenectomize if anemia is severe.

2. Hemoglobins with High O_2 Affinity – The prototype is hemoglobin Bethesda. The amino-acid substitution is near the α1-β2 interface, resulting in a tight binding of O_2. Release of O^2 to tissues is slow, resulting in inefficient tissue oxygenation. The end result is increased hemoglobin production due to high erythropoeitin levels.

Diagnosis is made by the *(a)* presence of familial erythrocytosis (polycythemia), *(b)* exclusion of other causes of polycythemia (polycythemia vera, cyanotic heart disease), *(c)* high red cell mass, *(d)* high arterial O_2 saturation, *(e)* a markedly LEFT shifted O_2 dissociation curve.

One should maintain hematocrit <70% by phlebotomy, to prevent high viscosity syndromes.

3. Hemoglobins with Low O_2 Affinity – The prototype is hemoglobin Kansas. The amino-acid substitution is also near the α1-β2 interface, but the result is low O_2 affinity. The hemoglobin picks up O_2 poorly

from the lungs, and high deoxyhemoglobin levels result, causing cyanosis.

Diagnosis is made by the O_2 dissociation curve, which is RIGHT shifted. The hemoglobin level and red cell mass are normal.

No specific management is necessary or effective. The cyanosis is relatively well tolerated if strenuous activities are avoided.

4. Hemoglobins M – The prototype is hemoglobin M Boston. The amino-acid substitution is near the heme pocket, close to the site of the Fe molecule. The mutant hemoglobin loses its ability to keep the Fe in its ferrous state, and the hemoglobin is constantly in the methhemoglobin state, Fe^{+++} and unable to carry O_2. The result is a chronic cyanotic state.

Diagnosis is made by a history of cyanosis since birth, with a normal O_2 saturation, brown discoloration of freshly drawn blood not changing with aeration, and by spectrophotometry to confirm presence of methhemoglobin. Electrophoresis also demonstrates the abnormal hemoglobin. No management is needed, as the amount of hemoglobin M is not sufficient to cause physiological derangements.

5. Hemoglobins C, D, E - These structural variants are synthesized at a lower rate than normal β chains and comprise less than half the total hemoglobin hemoglobin in heterozygotes. Heterozygous hemoglobin C (AC) results in mild target cells, but no anemia. Homozygous C (CC) produces a mild hemolytic anemia, significant red cell morphologic changes (target cells, hemoglobin crystals and microspherocytes), and mild splenomegaly. Heterozygous E (AE) causes a mild thalassemic phenotype with mild microcytosis and hypochromia. Homozygous E (EE) results in a moderate thalassemic phenotype, with significant hypochromia, microcytosis and a mild anemia. A combined E-β thalassemia inheritance results in a transfusion dependent thalassemic phenotype. Hemoglobin E is common in Southeast Asia and in certain areas of the Indian sub-continent .

USE OF TECHNOLOGIC ADVANCES

In the new millennium, numerous advances in genomics and other technologies have the promise of application to patients with hemoglobinopathies.

Prevention of new hemoglobinopathy births

1. Pre-marital screening and genetic counseling is easy and inexpensive. It should involve a non-directive genetic counseling for couples at risk and should target populations with a high prevalence of the hemoglobinopathy traits.
2. Prenatal diagnosis can be performed by chorionic villus sampling (CVS). Challenges include technical difficulties of the various methods used, as well as the expense. Furthermore, for safety and effectiveness, at risk pregnancies must present for antenatal diagnosis, ideally in the first trimester (8 to 14 weeks gestation). This requires awareness- raising programs in communities with a high prevalence rate of the genetic defect. The choice of a therapeutic abortion for affected pregnancies can be difficult due to cultural and religious issues. Research studies report a higher acceptance of prenatal diagnosis with a previously affected child. The costs of transfusion and chelation are prohibitive for families in developing countries. A useful strategy is universal screening for hemoglobinopathies among all pregnant women in high-risk areas of the country, initiated by mid-wives, who could also be trained to provide genetic counseling. Carrier women who are pregnant can then be referred for CVS to a local facility, and the sample sent to a tertiary laboratory for testing. Funding for this type of program, while expensive, can be cost-saving compared to the costs of treating the hemoglobinopathy.
3. Pre-conceptional diagnosis and implantation of normal embryos after *in vitro* fertilization is an alternative that is currently available in the West. It is extremely expensive and cannot be recommended routinely.
4. In-utero therapy using stem cell transplantation is an interesting and potentially exciting technology that would help couples at risk not opting for termination. It allows for the relative non-immunocompetent fetus to more easily accept the stem cell transplant without graft versus host disease. This is currently in early research trials.

Transfusion Safety Improvements

The mainstay of care of patients with hemoglobinopathies continues to be blood transfusion support. Transfusion safety is thus an important consideration, particularly the avoidance of transfusion transmitted infections. Donor screening has long been in place in developing countries. The Indian drug and cosmetics act of 1992 requires that blood collected for transfusions be tested negative for Hepatitis B, HIV, syphilis and malaria. However, the use of less sensitive test like reverse passive hemagglutination is permitted and the more sensitive ELISA techniques are often not used. Thus, donor screening may be ineffective in some areas of the country. Other pitfalls are the use of expired kits and reagents, and improper standard operation procedures. Numerous studies from India have indicated the risks of transfusion-transmitted infections.[6,7]

Simply changing from paid or "replacement" donors to voluntary donors can greatly reduce the prevalence of transfusion-transmitted infections. Such a change reported in the Japanese literature resulted in reduction of transfusion-transmitted hepatitis from 51% to less than 15% of.[8] Other strategies recommended are: reduction in the use of inappropriate blood transfusions, increased use of self-donated units that are stored and auto-transfused for elective surgical procedures, use of intra-operative

blood salvage techniques for surgical procedures, and effective, inexpensive testing of donors for infectious diseases. The development of central packaging of equipment, consumables, and data handling processes would be important in controlling quality of procedures, thus making blood transfusions safer. An independent national monitoring body should do monitoring of blood safety. Training of personnel who handle blood-banking facilities is also important, particularly for the storage and transport of blood. Recently published studies from India have shown a slight decrease in the seropositive rate of blood donors for HIV and HBV in the past 6 to 7 years. Seropositivity is consistently higher for "replacement" versus volunteer donors in these studies, suggesting the importance of encouraging an entirely volunteer donor system for the country.[9]

Stem cell Transplantation

An improved technique of stem cell transplantation using HLA matched sibling donors is now a curative procedure for the hemoglobinopathies. There is recent interest in non-myeloablative stem cell transplantation, which has less morbidity and leads to a chimerism in the recipient. This is still investigational in most countries.

CONCLUSION

An adaptation of technologic advances to the realities of health care in developing countries is a challenge. Awareness of genetic diseases, education and counseling of families at risk, while "low-tech", is crucial in avoiding the burden of these diseases to society.

REFERENCES

1. Bobhate SK, Gaikwad ST, Bhaledrao T. NESTROFF as a screening test for detection of β-thalassemia trait. *Indian J Pathol Microbiol* 2002; 45: 265-267.
2. Mehta BC. NESTROFT: a screening test for β-thalassemia trait. *Indian J Med Sci* 2002; 56: 537-545
3. Franchini M, Veneri D. Iron-chelation therapy: an update. *Hematol J* 2004; 5: 287-292.
4. Olivieri NF, Brittenham GM, McLaren CE *et al.* Long-term safety and effectiveness of iron-chelation therapy with deferiprone for thalassemia major. *N Engl J Med* 1998; 339: 417-423.
5. Wanless IR, Sweeney G, Dhillon AP *et al.* Lack of progressive hepatic fibrosis during long-term therapy with deferiprone in subjects with transfusion-dependent β-thalassemia. *Blood* 2002; 100: 1566-1569.
6. Singh H, Pradhan M, Singh RL *et al.* High frequency of hepatitis B virus infection in patients with beta-thalassemia receiving multiple transfusions. *Vox Sang* 2003; 84: 292-929.
7. Marwaha RK, Bansal D, Sharma S *et al.* Seroprevalence of hepatitis C and B virus in multiply transfused beta-thalassemics: results from a thalassemic day care unit in north India. *Vox Sang* 2003; 85 : 119-120.
8. Nishioka. Transfusion transmitted hepatitis B and C. *Vox Sang* 1996; 70.
9. Singh B, Verma M, Verma K Markers for transfusion-associated hepatitis in north Indian blood donors: prevalence and trends. *Jpn J Infect Dis* 2004; 57: 49-51.