PROFORMA FOR SUBMISSION OF PROJECT PROPOSALS ON RESEARCH AND DEVELOPMENT, PROGRAMME SUPPORT

(To be filled by the applicant)

PART I: GENERAL INFORMATION
1. Name of the Institute/University/Organization submitting the Project Proposal:
   Post Graduate Institute of Medical Education & Research, Chandigarh, India - 160012
2. State: Chandigarh

4. Name and designation of the Executive Authority of the Institute/University forwarding the application:
   Prof. Yogesh Chawla, Director, Post Graduate Institute of Medical Education & Research, Chandigarh

5. Project Title: Identification and characterization of target antigens on biliary epithelial cells and in blood in patients of biliary atresia using a proteomic approach.

6. Category of the Project: ☑ R&D ☐ Programme Support
7. Specific Area: Immunobiology
8. Duration: Three years
9. Total Cost (Rs.) …..Rs 41,79,299/-
10. Is the project Single Institutional or Multiple-Institutional (S/M)?: Single Institute
11. If the project is multi-institutional, please furnish the following: Single Institute
    Name of Project Coordinator:

    Affiliation:

    Address:

    ………………………………………………………………………………………………………………………………………………………………………
12. **Scope of application indicating anticipated product and processes**  
The study aims to identify unknown target antigens in biliary atresia (BA) which are responsible for immune mediated destruction of biliary tracts. The proteomic approach and animal experiments used in the study will help in the characterization and confirmation of unknown target proteins as etiological factors. The study will enhance our understanding of disease pathogenesis and may help in pinpointing an early diagnostic biomarker and planning new therapeutic modalities for BA.

13. **Project Summary**  
Biliary atresia (BA) is life threatening and most common cause of obstructive jaundice in infancy with limited therapeutic options. It is a progressive inflammatory fibrosclerosing lesion of the bile ducts that finally leads to biliary cirrhosis. The dilemma is that all therapeutic attempts to cure the disease provide only short term benefits and the disease maintains a progressive course. These treatment hurdles are persisting because the exact etiology of the disease is unclear. To improve treatment results, elucidation of the underlying mechanism of destruction of biliary tract leading to BA is crucial. Morphological studies on bile duct tissue report a sclerosing inflammatory reaction which leads to destruction of previously well formed bile ducts. However, what triggers this immune mediated reaction and consequent obliteration of the biliary tree is poorly understood. It has been suggested that unknown antigens, either of biliary origin or circulating in the blood may be the targets of this immune or autoimmune mediated response. Therefore, identification and characterization of these unknown antigens may be of great value in understanding the pathogenesis of the disease. In the present study a proteomic approach will be used to identify these unknown target antigens both on the biliary epithelial cells as well as those circulating in the blood. Further, the identified protein will be injected in to an experimental animal model to induce BA. This will be followed by histopathological examination for the confirmation of role of identified proteins as etiological factors in BA. The study will be useful for the development of an early diagnostic biomarker and in designing therapies targeting that antigen. To the best of our knowledge, not a single study using proteomics and experimental animal models to isolate and confirm target antigens on cultured biliary epithelial cells and serum in BA has been published till date.
PART II: PARTICULARS OF INVESTIGATORS

(One or more co-investigators are preferred in every project. Inclusion of co-investigator(s) is mandatory for investigators retiring before completion of the project)

Principal Investigator:
14. Name: Yashwant Kumar
Date of Birth: 03-11-1974 Sex (M/F): Male
Designation: Assistant Professor
Department: Immunopathology

Institute/University:
Post Graduate Institute of Medical Education & Research, Chandigarh, India - 160012

Address:
Department of Immunopathology, Post Graduate Institute of Medical Education & Research, Chandigarh, India, 160012

Telephone: 0172-2745191; Mob 8437220033  Fax: 0172-2744401
E-mail: dryashwant@ymail.com

Number of research projects being handled at present: None

Co-Principal Investigator
15. Name: Prof. Ranjana Walker Minz
Date of Birth: 04-07-1959  Sex (M/F): Female
Designation: Professor & Head
Department: Immunopathology

Institute/University: Post Graduate Institute of Medical Education & Research, Chandigarh, India - 160012

Address:
Department of Immunopathology, Post Graduate Institute of Medical Education & Research, Chandigarh, India, 160012

Telephone: 0172-2745193  Fax: 0172-2744401  E-mail: rwminz.minz@gmail.com

Number of research projects being handled at present: 03
Co-Principal Investigator
15. Name: B R Thapa
Date of Birth: 13-03-1953   Sex (M/F): Male
Designation: Professor
Department: Pediatric Gastroenterology
Institute/University: Post Graduate Institute of Medical Education & Research, Chandigarh, India - 160012
Address: Department of Pediatric Gastroenterology, Post Graduate Institute of Medical Education & Research, Chandigarh, India, 160012
Telephone: 0172-2746607   Fax: 0172-2744401  E-mail: brthapa1@yahoo.co.in
Number of research projects being handled at present:  None

Co-Investigator
15. Name: Dr Veena Dhawan
Date of Birth: 17.03.1956   Sex (M/F): Female
Designation: Additional Professor
Department: Experimental Medicine & Biotechnology
Institute/University: Post Graduate Institute of Medical Education & Research, Chandigarh, India - 160012
Address: Department of Experimental Medicine & Biotechnology, Post Graduate Institute of Medical Education & Research, Chandigarh, India, 160012
Telephone: 0172-2745235   Fax: 0172-2744401  E-mail: veenad2001@yahoo.com
Number of research projects being handled at present:  02

Co-Investigator
15. Name: Ravi Prakash Kanojia
Date of Birth: 20.04.1976   Sex (M/F): Male
Designation: Assistant Professor
Department: Pediatric surgery
Institute/University: Post Graduate Institute of Medical Education & Research, Chandigarh, India - 160012
Address: Department of Pediatric surgery, Post Graduate Institute of Medical Education & Research, Chandigarh, India, 160012
Telephone: 0172-2745339   Fax: 0172-2744401  E-mail: drravikanojia@yahoo.com
Number of research projects being handled at present:  None
16.1 **Origin of the proposal**

Biliary atresia (BA), a condition unique to infancy, is the end result of a destructive, idiopathic and inflammatory process affecting both intrahepatic and extrahepatic bile ducts, leading to fibrosis and obliteration of biliary tract and to biliary cirrhosis. It constitutes 30% of the neonatal and infantile cholestatic disorders. The symptoms of BA appear within first 3 months of life in the form of conjugated hyperbilirubinemia, acholic stools, and hepatomegaly. There is a female preponderance and the disease has been noted to be commoner in Asian countries (1 of 8,000 live births) than in European population (1 of 18,000 live births). Laboratory evaluation typically shows conjugated hyperbilirubinemia (>20% of total serum bilirubin) with elevation of serum concentrations of hepatocellular enzymes like aspartate aminotransferase [AST], alanine aminotransferase [ALT] and canalicular enzymes i.e. alkaline phosphatase, $\gamma$-glutamyltranspeptidase [GGT].

Despite early diagnosis and prompt surgical intervention with the Kasai procedure, BA continues its progressive course. If BA is diagnosed before 2 months age, Kasai procedure (a porto-enterostomy by the excision of the extrahepatic biliary remnants and anastomosis to a jejunal loop) done to re-establish a patent conduit for bile flow from the liver to the small bowel generally restores bile flow in 70%-80% of patients. However, the disease ultimately ends into end-stage cirrhosis. Post Kasai procedure, the survival rates are only 20%-30% of these patients and eventually 70% to 80% of children with BA require liver transplantation. Liver transplantation is therapeutically effective and increases 5 year survival rate to 75%-90% but is not a final solution to this unique disorder as there is often shortage of donors along with risk and complications of life-long immunosuppression. Moreover facilities of liver transplantation exist in few centers only.

Consequently, complete success in BA treatment is not yet realized. In our country post Kasai procedure 1 year survival is 20%-30%. Moreover the liver transplantation facilities are not freely and easily available.

Two major forms of BA are recognized based on the presumed timing of the obliteration of the lumen of the extrahepatic bile duct: i) the fetal or embryonic or prenatal form and ii) perinatal or postnatal form. The ‘fetal form’ occurs in 10%-20% of cases only, presents earlier in infancy and is associated with other congenital anomalies like splenic malformation (e.g., polysplenia, asplenia, double spleen), intestinal malrotation, absent inferior vena cava, situs inversus, cardiac anomalies (e.g., ventricular septal defect, atrial
septal defect, hypoplastic left heart), preduodenal portal vein and annular pancreas.\textsuperscript{6,7} Because of these associated anomalies, a defect in morphogenesis of the biliary tree has been suggested which is assumed to be caused by abnormal expression of genes that determine laterality of thoracic and abdominal organ development.\textsuperscript{3} The more common ‘perinatal or postnatal form’ of BA is responsible for 80\%-90\% of cases and is believed to occur at or following birth with progressive postnatal destruction of a biliary tree that developed normally during embryogenesis. Despite several years of sincere efforts, pathogenesis of the obliteration of the biliary tree remains elusive. In recent years multiple etiologies in the development of BA have been proposed (table).\textsuperscript{8}

\begin{table}
\centering
\caption{Proposed etiologies of BA\textsuperscript{8}}
\begin{tabular}{l}
Infectious - reovirus, rotavirus, retrovirus, cytomegalovirus, human papilloma virus, etc  
Immune dysregulation  
Autoimmune mechanism  
Vascular lesion/arteriopathy  
Defective morphogenesis  
Inherited mutations  
\hspace{0.5cm} Genes, associated with polysplenia and asplenia syndromes (e.g. $CFC1$)  
\hspace{0.5cm} Ductal plate malformation (e.g., $HNF6$)  
\hspace{0.5cm} Jagged 1  
\hspace{0.5cm} Somatic mutations  
\hspace{0.5cm} Modifier genes  
Toxin exposure
\end{tabular}
\end{table}

Inherited mutations are not believed to be responsible for BA (except for some cases of polysplenia syndrome-associated BA) because human leukocyte antigen (HLA)-identical twins discordant for BA have been described, and recurrence of BA within the same family is exceedingly rare.\textsuperscript{9-10} Bile duct injury at the time of vulnerable stage of human biliary development (between 11 and 13 weeks of gestation) and extravasation of bile with its toxic detergent constituents into the submucosa may also elicit a secondary inflammatory sclerosing process.\textsuperscript{11} However, this cause is not explained as ductal obstruction remains progressive even after a “successful” porto-enterostomy that establishes bile flow. Epidemiologic studies also support a possible infectious etiology to BA. There has been continued demonstration of seasonal clustering of cases, suggesting environmental exposure to an infectious agent.\textsuperscript{12} In addition, several models of viral infection in newborn mice produce lesions similar to BA.\textsuperscript{6} However results in human studies are confounding.\textsuperscript{13-17}
A “multihit” hypothesis for the pathologic process of BA has also been proposed. According to this a viral or toxic insult to biliary epithelium leads to newly expressed or altered antigens on the surface of bile duct epithelia, which, in the proper genetically determined immunologic milieu, are presented by macrophages to T lymphocytes. The cytotoxic T cells then elicit a Th1 cellular response causing bile duct epithelial injury, eventually resulting in fibrosis and occlusion of the extrahepatic bile duct (figure 1).

Figure: Proposed model of viral/unknown antigens induced auto reactivity leading to immune mediated destruction of bile duct epithelium in patients with BA. The initial injury to the bile duct epithelial cells from a virus or other potential insult leads to new appearance of previously sequestered self-antigens or self antigens altered by caspases induced during bile duct epithelial cell apoptosis, inciting further ductal damage through auto reactive T lymphocyte mediated inflammation. Initial T-cell activation specific to the target antigen leads to interferon $\gamma$ stimulation of macrophages with release of nitric oxide (NO), reactive oxygen species ($O_2^-$), and tumor necrosis factor (TNF) and subsequent epithelial cell death through apoptotic or necrotic pathways. Previously sequestered or altered bile duct epithelial antigens released from this initial injury are now presented to auto reactive T cells, causing further activation of this immune cascade and progressive destruction of bile duct epithelium.

Earlier studies on bile duct remnants removed at surgery and from serial sectioning and reconstruction of surgical and post mortem liver specimens reported that BA in most cases
arises from a sclerosing inflammatory process affecting previously formed bile ducts.\textsuperscript{19,20} What triggers this intense inflammatory reaction and consequent obliteration of the biliary tree is poorly understood but biliary epithelial cells (BECs) are targets of this immune or autoimmune mediated response.\textsuperscript{21} Understanding the intricacies of the inflammatory mechanisms culminating in bile duct injury are crucial to the future development of therapies aimed at halting the ongoing biliary tract destruction found in BA. The study therefore is planned to identify these triggering factors linked with the etiopathogenesis of BA.

16.2 (a) Rationale of the study supported by cited literature

BA is life threatening and most common cause of obstructive jaundice in infancy with limited therapeutic options. It is a progressive inflammatory fibrosclerosing lesion of the bile ducts that finally leads to biliary cirrhosis. The treatment hurdles for BA are persisting because the exact etiology of the disease is unclear. Recent studies indicate that the ongoing ductal destruction could be immune mediated. Although several experts have proposed that BA is an "autoimmune" disorder there is sparse evidence of autoimmunity to support this contention. The use of DNA microarray to study BA reveals possible genetic induction of proinflammatory immunity in this disorder. This immune-mediated reaction is likely to be triggered by an antigen of unknown origin, either microbial or a self-antigen, that could be presented to T cells by dendritic cells. The present study aims to identify these unknown target antigens which modulate immune system in BA leading to immune mediated destruction of biliary tracts. These target antigens if identified will help not only explore the etiology of the disease but also may be used for designing of a long awaited diagnostic biomarker. In addition, therapies targeting these antigens may also be developed and used in future for treatment of BA.

(b) Hypothesis

To understand the etiopathogenesis of BA many hypotheses have been proposed but nothing is definite. Though the factors like genetic and infections have been suggested to be associated with BA however, the etiology of BA remains a mystery. Recently an immune mediated etiology has been suggested on the basis of inflammation related destruction of biliary tree. It is possible that the immune cells are activated by some unknown antigens which either persist or leave certain long lasting immunoactivator thereby leading to a progressive course of the disease. In this study a proteomic approach
will be used to identify unknown target antigens followed by injecting them in mice model and induction of experimental BA in vivo for their evaluation as etiological factors.

We postulate that if these unknown antigens are isolated, identified and if they can induce experimental disease in the animal it will open a new vista for understanding the disease, designing a diagnostic biomarker and development of new therapeutic options for BA.

(c) Key questions

1) Why in majority of cases changes in the biliary duct occur after birth? If it is acquired then what are the etiological factors?
2) Why there is an inflammation related destruction of bile ducts? Is it due to some infectious antigen or auto antigen? If yes then how to detect them?
3) Will proteomic approach be successful in identifying these antigens? Will the identified antigens successfully induce experimental BA in animals?

16.5 Current status of research and development in the subject (both international and national status)

BA is a devastating disease of infants, invariably leading to cirrhosis, end-stage liver disease, and death if untreated within 2 years of life. In the recent years many studies, both human and animal, have been carried out to explore the etiology of BA (nearly 50 publications on pubmed after the year 2005). Most of these have been done to establish the link between one or more viruses and BA. Some also tried to find out certain polymorphic genes or other genetic factors. Various immunologic factors have also been studied and some of them have come with positive outcome as well i.e. role of both innate and adaptive immunity with elevation of certain cytokines like IFN-γ, TNFα, IL-2, IL-6, IL-8, CXC etc. However target antigens leading to activation of immune response have not been fully recognized. Till date, there is no published study using proteomic approach to detect target antigens both in blood and on biliary epithelial cells. In a study by Lee et al., 2D gel electrophoresis and mass spectrometry was used to analyze serum samples in 6 children with BA and they found increased levels of immunoglobulin kappa light chain and reduced levels of apolipoprotein A-I and C-II, haptoglobin α2 and β chain, and transthyretin (prealbumin) however, the identified proteins were not further studied for their etiologic role. Moreover biliary epithelial cells antigens were also not utilized for analysis.

In Indian subcontinent also, no similar attempt to explore the unknown target antigens in BA has been made so far.
References


16.6 The relevance and expected outcome of the proposed study

Despite extensive research the cause(s) of BA remain elusive. There are enough evidences that, in BA there is progressive destruction of both intra as well as extra hepatic biliary tree which is mediated by immune cells. The antigens triggering this immune response however are not known. These may be of microbial origin or even auto antigens on biliary epithelial cells. It is possible to identify and characterize these target antigens using a proteomic approach. Though proteomic studies to identify unknown antigens have been done in other idiopathic disorders i.e. autoimmune rheumatologic disorders, pulmonary arterial hypertension, giant cell arteritis etc., a systematic approach has not been tried in BA.

The study will reveal unknown target antigens against which immune cells are activated which in turn lead to progressive destruction and sclerosis of biliary apparatus. This will help in understanding of disease pathogenesis. Also some of these target antigens related results may be used in designing of future diagnostic biomarkers or even in development of new therapeutic modalities.

16.7 Preliminary work done so far

The one of the Co-Principal investigators of the project has ≥25 years of experience in the field of immunopathology along with considerable expertise in proteomics, animal studies and she has been constantly working on antigen detection methods for last few years. Recently a PhD work on proteomics has been completed under her guidance. Moreover she has been constantly working on autoimmune hepato-biliary diseases for past several years. The other Co-Principal Investigator is a clinician with ≥30 years of experience in diagnosis and management of BA. He also been working to explore the causative factors of BA and has published many articles related to the disease. One Co-Investigator is a scientist already working on protein extraction and proteomics. The other Co-Investigator is a surgeon devoted particularly to biliary atresia and its surgical management. Although this will be first project on BA for the principle investigator however he is a budding, hardworking and enthusiastic immunopathologist and has recently worked on autoimmune disorders. The research team therefore, has a blend of experience, intent and reverence to the related subject and would like to work on detection of target antigens and establish their roles in BA.
17. **Aim:**
To identify and characterize target antigens in blood and on biliary epithelial cells in patients of BA by proteomic approach.

**Objectives:**
For isolation of new target antigens, first biliary epithelial cells will be cultured and serum will be separated from blood of patients with BA and proteins will be extracted and determined using standard methods. The isolated protein will be subjected to first and second dimension gel electrophoresis. The protein spots thus obtained will be quantified using appropriate software (PD QUEST) and excised. Thereafter, these proteins will be analyzed by Matrix Assisted Laser Desorption Ionization and time of flight (MALDI-TOF) which will be outsourced. The final product identified will be matched with the type of protein using proteomic database software. Finally the target antigen(s) obtained will be injected into 20 young albino mice (an experimental model used for BA). Four mice (2 males and two females) will be sacrificed immediately after antigen injection. Other sets will be sacrificed at 2, 3, 4 and 6 weeks interval. Induction of the disease will be confirmed by clinical and histopathological evaluation (Figure 2).
18.1 **Work plan**

**Patient selection and sample collection**

A total of 20 patients of BA with age <12 years will be recruited from the division of Pediatric Gastroenterology & Pediatric Surgery, PGIMER, Chandigarh. About 3 ml venous blood will be drawn in a plane vacutainer from these children before they undergo surgery for porto-enterostomy (Kasai procedure). The excised remnants of biliary tract will be stored immediately at 4°C in Dulbecco’s modified Eagle medium (DMEM). Ten healthy age and sex matched controls will also be recruited for comparison and 3 ml of blood sample will be collected from them. These children will be those visiting to pediatric gastroenterology unit for routine follow up after subsidence of minor illnesses i.e. gastroenteritis and having normal hematological and biochemical parameters. These samples will be stored and processed in the same manner to those of patient’s samples. A written informed consent will be obtained both
from cases and control subjects/their guardians for the study. In addition, protein extracts from cholangiocarcinoma cell line will also be used as control for comparison from patient’s biliary tissue. These cell lines will be purchased from a cell line repository i.e. National Centre for Cell Science, Pune. The cell extracts will be obtained and processed in a similar manner to that of patient’s tissue.

**Inclusion criteria:**

- Diagnosed cases of BA based on:
  - Suggestive clinical history i.e. acholic stools
  - Ultrasonography, Mebrofenin hepato-biliary scan (HIDA scan) and per-operative cholangiogram findings
  - Histopathology of scrapping and liver biopsy

**Exclusion criteria:**

- Exclusion of other causes of neonatal cholestasis i.e. Neonatal hepatitis, Choledochal cyst, Congenital hepatic fibrosis, patent vitalo-intestinal duct (PVID), α-1 antitrypsin deficiency etc.
- Hemolysed blood samples
- Patient/guardian not willing for participation to the study

**Biliary epithelial cell (BEC) culture**

The porta hepatis part of the bile duct remnants obtained during operation will be immediately stored at 4°C in Dulbecco’s modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) until seeding.

The obtained bile duct remnants will be cut into small pieces, the epithelium will be stripped off with the help of a sterile surgical blade and placed on Petri dishes. After adding culture medium, growth factors, antibiotics and antifungal medication the cells will be grown at 37°C in 5%CO₂. The dishes will be periodically observed and the proliferated BEC will be isolated. If required passage culture of subcultured BEC will be performed.²⁵

After proliferation the cells will be examined under a phase contrast microscope and immunostained for CK7 antibody (Sigma) to confirm their biliary origin (BEC should be positive for CK7).²⁶

**Protein extraction and protein estimation from biliary tissue & blood**

After morphological and immunocytochemical (ICC) confirmation, the cells will be isolated, washed and dissolved in a standard cell lysis buffer and total proteins will be
extracted using protein extraction kit (G-Biosciences, USA) and the supernatant will collected. The concentration of total protein will be determined using a Protein estimation assay kit as per instruction manual (G-Biosciences, USA). The protein concentration will be adjusted equal for both patients and control tissues, deep frozen in liquid nitrogen and stored at -80°C.

The whole blood sample will be centrifuged immediately after it is received and serum obtained will be stored at -80°C. At the time of analysis, the stored serum will be thawed and 200 µl will be added to 1 ml of Trizol reagent (Sigma, USA). About 200 µl of chloroform will be added followed by 15 min incubation at room temperature (RT). Thereafter it will be centrifuged at 12000 rpm for 15 min at 4°C. The organic phase will be transferred to fresh eppendorf and ispropanolol (1.5 ml/1ml of Trizol reagent) will be added. After 10 min incubation at RT it will be centrifuged at 12000 rpm for 10 min at 4°C. The supernatant will be removed and after washing thrice with 0.3M Guanidium Hydrochloride in 95% Ethanol (2 ml 0.3M Guanidium Hydrochloride/95% Ethanol per 1 ml of Trizol reagent) the pellet will be air dried and dissolved in PBS and proceed for desalting by dialysis. The protein concentration will be adjusted equal for both patients and control tissues, deep frozen in liquid nitrogen and stored at -80°C.

**First Dimension Gel Electrophoresis**

Sample will be prepared in a rehydration buffer and 300 µl will be added as a line along back edge of a channel in a rehydration/equilibration tray. IPG strip (Bio-Rad) of suitable length will be chosen and the plastic leaf will be stripped out carefully. The strip will be placed in the rehydration/equilibration tray lane over the sample and 2-3 ml of mineral oil will be overlaid over the strip to prevent evaporation during the rehydration process. The rehydration/equilibration tray will be covered with plastic lid and kept sitting on a level bench overnight (11-16 hrs) to rehydrate the IPG strip. The cover from rehydration/equilibration tray containing the IPG strip will be removed and tip of strip will be blotted on a piece of filter paper to allow the mineral oil to drain. The IPG strip will then be transferred to the corresponding channel in the isoelectric focusing (IEF) tray according to its polarity. The tray will be covered and placed in to the IEF cell. After electrophoresis run the strip will be kept at -80º C for overnight.

**Second Dimension Gel Electrophoresis**

Five ml of equilibration buffer-1 will be added with dithiothretiol (DTT) to an equilibration/rehydration tray. The mineral oil on IPG strip will be removed by filter
paper and the strip (gel side up) will be transferred to the equilibration tray. The tray will be placed on a rocker shaker and gently shaken for 10 min followed by incubation for 10 min. The equilibration buffer-1 will be discarded and 5 ml of equilibration buffer-2 will be added with iodoacetamide to the strip. Tray will be placed on rocker shaker and gently shaken for 10 min followed by incubation for 10 min. Equilibration buffer-2 will be discarded by decanting and moisture will be drained from the equilibration strip by soothing it over a filter paper.

Sodium Dodecyl Sulphate (SDS) run
Resolving gel solution of 12% will be prepared and poured between the plates of the cassette. It will get polymerized after 30-40 min. Gel layer will be filled with water (milliQ) immediately to prevent air contact and to make sure it settles evenly. Stacking gel solution will be prepared and poured over the polymerized resolving gel in the cassette. Strip will be dipped in the SDS electrophoresis buffer for lubrication and placed in between the two glass plates of the cassette. Top of the plates will be sealed carefully with agar sealing solution avoiding any air bubbles. Tank will be filled with electrophoresis buffer and gel cassette will be placed in the 2D gel running assembly. Gel will be run initially at 16 mA for 15 min followed by at 25 mA till the dye reaches the bottom of the gel. Gels will be silver stained using the protocol compatible with mass spectrum analysis. Image analysis will be carried out using appropriate software for the differential expressed protein spots (PD QUEST).

Identification and characterization of proteins spots obtained from 2D PAGE analysis of cell and serum samples by Matrix Assisted Laser Desorption Ionization and time of flight (MALDI-TOF)
Gel will be rinsed 2 times for 10 min in milliQ water and transferred on alcohol wiped transparency. The spot/band of interest will be cut and gel plugs will then be transferred into clean eppendorf tubes. These silver stained spots will be washed with HPLC water and destaining solution-II for 10-15 min with a continuous vortexing. Thereafter Gel pieces will be dehydrated by adding acetonitrile and incubated for 5 min at room temperature (RT). The reduction solution will be added to the tubes containing gel pieces and incubated at 56°C for 30 min. After discarding reduction solution, alkylation buffer solution will be added to the gel pieces and incubation will be done in dark for 30 min at RT. After discarding alkylation buffer the gel pieces will be washed by adding destaining solution-I. After addition of acetonitrile gel pieces will be
allowed to air dry. Once dried gel pieces will be subjected to tryptic digestion by addition of 20 ng/μl of trypsin solution to the tube followed by incubation at 37° C for 16 hrs. Finally peptide extraction solution will be added and tubes will be sonicated in a water bath sonicator.

Sample for MALDI-TOF analysis will be prepared using dried droplet method. For this 1μl peptide solution (peptide extracts after tryptic digestion) and 1 μl of a suitable matrix e.g. alpha-cyano hydrocinnamic acid (HCCA) in 1:2 v/v of acetonitrile (ACN): 0.1% TFA (trifloro acetic acid) will be mixed together and 1μl of this mixture will be spotted on a MALDI target plate and allowed to air dry at RT. Peptide calibration standard (Bruker Daltonics, USA) will also be prepared in the same way. MALDI target plate will be loaded in to Ultraflex MALDI-TOF for subsequent peptide spectra acquisition and analysis. A laser power of 337 nm wavelength will be used for ionization of the samples spotted on the target and peptide peaks will be calibrated with peaks obtained from the peptide calibration standard. After peptide spectra are obtained, MS analysis will be carried out using flex analysis software (version 2.2, Bruker). Subsequent MS data analysis will be carried out using Biotools software (version 2.2, Bruker Daltonics) and MASCOT search engine (Matrix Science) against the NCBI database.

Animal experiment

Antigen preparation prior to injection
Polyacrylamide gel will be carefully trimmed and target antigens will be separated. The antigen will be emulsified in PBS and complete Freunds reagent (CFA, Sigma, USA) will be used as systemic adjuvant.

Induction of disease
Thirty young Swiss albino mice (15 male and 15 female) will be obtained and kept under standard conditions in laminar-flow cages with free access to water and standard mouse feed. BA will be induced by 0.2 ml intra peritoneal injection of the prepared antigen. Six mice (3 male and 3 female) will be sacrificed immediately after injecting the antigen solution. The other sets (each of 6) will be sacrificed at 2, 3, 4 and 6 weeks interval.

The mice will be assessed daily for signs of cholestasis (jaundice of the non-fur-covered skin, colour and quality of stools, appearance of bilirubin in the urine). At specified time each set of mice will be sacrificed by giving an intra peritoneal injection of sodium pentobarbital (50 mg/kg) followed by cardiac puncture. Immediately after
the animal’s death, a midsection laparotomy will be carried out. Hepato-biliary apparatus and a part of duodenum will be examined (common bile duct, atretic/hydropic gallbladder and liver parenchyma) and sampled. The tissue will be fixed in 10% buffered formalin solution for a period of 24-48 hours after which they will be paraffin-embedded. Five micrometer thick histological slices from the paraffin blocks will be obtained on glass slides and will be stained with hematoxylin and eosin stain (HE). The HE stained slides will be analyzed under light microscopy. The histological characteristics (ductule proliferation and inflammatory infiltrate in the portal space, bile plugging of interlobular bile ducts) will be assessed at each phase of the experiment. The semi-quantitative measurements will be carried out through scores from 0 to 4, being (0), the absence of the finding; (1) a mild alteration, (2) a moderate alteration, (3) intense alteration, and (4) as the very intense/maximum response.

18.2 **Connectivity of the participating institutions and investigators**

Single Institute

18.3 **Alternate strategies**

Biliary epithelial cells will be cultured to enhance antigen retrieval however if cells culture could not be performed direct analysis from cell lysates will be performed.

19. **Timelines:** (Please provide quantifiable outputs)

<table>
<thead>
<tr>
<th>Period of study</th>
<th>Achievable targets</th>
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<tbody>
<tr>
<td>6 Months</td>
<td>Recruitment of research assistant, Procurement of equipments, consumables and reagents</td>
</tr>
<tr>
<td>12 Months</td>
<td>Sample collection, cell culture, protein purification and immunoblotting</td>
</tr>
<tr>
<td>18 Months</td>
<td>Identification and characterization of target antigens by 2D gel electrophoresis and MALDI-TOF</td>
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<tr>
<td>24 Months</td>
<td>Procurement and maintenance of animals, induction of BA in animal models and its confirmation by histopathology</td>
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<tr>
<td>30 Months</td>
<td>Collection and analysis of data</td>
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<tr>
<td>36 Months</td>
<td>Compilation of results and submission of report</td>
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20. Name and address of 5 experts in the field

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Name</th>
<th>Designation</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dr Seema Alam</td>
<td>Professor &amp; Head</td>
<td>Department of Pediatric Hepatology, Institute of Liver &amp; Biliary Sciences, New Delhi</td>
</tr>
<tr>
<td>2</td>
<td>Dr Anupam Sibal</td>
<td>Head of the Unit</td>
<td>Department of Pediatric Gastroenterology, Apollo Hospital, New Delhi</td>
</tr>
<tr>
<td>3</td>
<td>Dr Sarath Gopalan</td>
<td>Senior Consultant</td>
<td>Pediatric Gastroenterology &amp; Hepatology, Pushpawati Singhania Research Institute, Delhi</td>
</tr>
<tr>
<td>4</td>
<td>Dr Sutapa Ganguli</td>
<td>Professor &amp; Head</td>
<td>Pediatric Gastroenterology Unit, Department of Pediatric Medicine, Nilratan Sircar Medical College and Hospital, Kolkata</td>
</tr>
<tr>
<td>5</td>
<td>Dr. B.Bhaskar Raju</td>
<td>Professor &amp; Head</td>
<td>Pediatric Gastroenterology Department, Institute of Child Health &amp; Hospital for Children, Chennai</td>
</tr>
</tbody>
</table>
**PART IV: BUDGET PARTICULARS**

**Budget (In Rupees)**

**A. Non-Recurring (e.g. equipments, accessories, etc.)**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Item</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CO\textsubscript{2} incubator</td>
<td>5,00,000</td>
<td></td>
<td></td>
<td>5,00,000</td>
</tr>
<tr>
<td>2</td>
<td>Laminar flow hood</td>
<td>5,00,000</td>
<td></td>
<td></td>
<td>5,00,000</td>
</tr>
<tr>
<td>3</td>
<td>Micro centrifuge with rotor attachments</td>
<td>1,00,000</td>
<td></td>
<td></td>
<td>1,00,000</td>
</tr>
</tbody>
</table>

Sub-Total (A) = 11,00,000

**B. Recurring**

**B.1 Manpower**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Position No.</th>
<th>Consolidated Emolument</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Research Assistant</td>
<td>19,481</td>
<td>2,23,772</td>
<td>2,23,772</td>
<td>2,23,772</td>
<td>7,01,316</td>
</tr>
<tr>
<td>2</td>
<td>Animal/ lab attendant</td>
<td>9,896</td>
<td>1,18,752</td>
<td>1,18,752</td>
<td>1,18,752</td>
<td>3,56,256</td>
</tr>
</tbody>
</table>

Sub-Total (B.1) = 29,377 + 3,42,524 + 3,42,524 + 3,42,524 = 10,27,572

**B.2 Consumables**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Item</th>
<th>Quantity</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Biliary epithelial cell culture media, growth factors, cell lines, CK7 immunostain and reagents</td>
<td>Variable</td>
<td>1,50,000</td>
<td>1,00,000</td>
<td></td>
<td>2,50,000</td>
</tr>
<tr>
<td>2</td>
<td>Protein purification kit, protein estimation kit &amp; reagents</td>
<td>1 kit each</td>
<td>1,00,000</td>
<td>1,00,000</td>
<td></td>
<td>2,00,000</td>
</tr>
<tr>
<td>3</td>
<td>2D gel electrophoresis strips and reagents</td>
<td>Variable</td>
<td></td>
<td>3,00,000</td>
<td>2,00,000</td>
<td>5,00,000</td>
</tr>
<tr>
<td>4</td>
<td>MALDI-TOF analysis</td>
<td>-</td>
<td>-</td>
<td>2,00,000</td>
<td>2,00,000</td>
<td>4,00,000</td>
</tr>
<tr>
<td>5</td>
<td>Animal cages &amp; diet, plastic &amp; glassware, histopathological &amp; other reagents</td>
<td>-</td>
<td>1,00,000</td>
<td>1,50,000</td>
<td>1,00,000</td>
<td>3,50,000</td>
</tr>
</tbody>
</table>

Sub-Total (B.2) = - + 3,50,000 + 8,50,000 + 5,00,000 = 17,00,000

**Other items**

<table>
<thead>
<tr>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.3 Travel</td>
<td>30,000</td>
<td>25,000</td>
<td>25,000</td>
</tr>
<tr>
<td>B.4 Contingency</td>
<td>50,000</td>
<td>50,000</td>
<td>50,000</td>
</tr>
<tr>
<td>B.5 Overhead (3% institute charges on total project cost)</td>
<td>56,175</td>
<td>38,026</td>
<td>27,526</td>
</tr>
</tbody>
</table>

Sub-total of B = 10,57,572 + 17,00,000 + 80,000 + 1,50,000 + 1,21,878 = 27,49,699

Grand Total (A + B) = 11,00,000 + 30,79,299 = 41,79,299
Justification

1. **Staff:** Research assistant will be required to carry out research work. Lab attendant will help in pre and post analytic lab work, transport of samples/specimens and in animal care.

2. **Equipments:** CO$_2$ incubator and laminar flow hood will be required for cell culture. Micro centrifuge will be used for separation of cellular components from specimens. None of these equipments are available as spare equipment in the department for work related to the study.

3. **Travel:** Travel grant will be utilized for attending various conferences and CMEs on topics related to the project, travel connected with procurement of chemicals/equipments. The personnel who are working in the project shall also present the results of their work from this project in various conferences.

4. **Contingency:** The contingency will be required for purchasing the plastic ware; trays etc. required for collect and store the samples. It will also be used for acquisition of books and documents of relevance to the research topic in case these are not available in the library, computer utilities and charges for analysis of data (computer charges), publication charges/reprints/off-prints of research papers published as an outcome of the research.

5. **Overheads:** The 3% overheads charges on total budget must be given to PGIMER to perform financial and administrative activities of the project.
PART V: EXISTING FACILITIES

Resources and additional information

1. Laboratory & Manpower:
Department of Immunopathology provides advanced diagnostic services to its patients and is actively involved in various research projects in different aspects of Immunology. There are more than 20 PhD and Msc students working under the guidance of experienced senior faculty members. The department also trains more than 20 MD students. The major labs, each having adequate staff, and important equipment available in the department are:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HLA lab</td>
</tr>
<tr>
<td>2</td>
<td>Autoimmunity lab</td>
</tr>
<tr>
<td>3</td>
<td>Molecular lab</td>
</tr>
<tr>
<td>4</td>
<td>Electrophoresis lab</td>
</tr>
<tr>
<td>5</td>
<td>HIV lab</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Equipments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agarose gel electrophoresis apparatus</td>
</tr>
<tr>
<td>2</td>
<td>Semi automated electrophoresis instrument</td>
</tr>
<tr>
<td>3</td>
<td>Real time PCR machine</td>
</tr>
<tr>
<td>4</td>
<td>Thermocycler</td>
</tr>
<tr>
<td>5</td>
<td>Nephelometer</td>
</tr>
<tr>
<td>6</td>
<td>ELISA reader</td>
</tr>
<tr>
<td>7</td>
<td>-80°C C ultra low refrigerators</td>
</tr>
<tr>
<td>8</td>
<td>BOD incubator</td>
</tr>
<tr>
<td>9</td>
<td>Fluorescence &amp; inverted microscopes</td>
</tr>
<tr>
<td>10</td>
<td>Ultra centrifuge</td>
</tr>
<tr>
<td>11</td>
<td>Cold Room</td>
</tr>
</tbody>
</table>

2. Other resources such as clinical material, animal house facility, glass house. Experimental garden, pilot plant facility etc.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Infrastructural Facility</th>
<th>Yes/No/ Not required Full or sharing basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Workshop Facility</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Water &amp; Electricity</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>Laboratory Space/ Furniture</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>Power Generator</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>AC Room or AC</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>Telecommunication including e-mail &amp; fax</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>Transportation</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>Administrative/ Secretarial support</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>Information facilities like Internet/Library</td>
<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>Animal/ Glass House</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Information to participants and consent form

PROTOCOL NO:

SPONSOR: Department of Biotechnology, New Delhi

INVESTIGATOR (Principal and at least one Co-Investigator):

Dr Yashwant Kumar, Assistant Professor, Immunopathology (PI),
Dr Ranjana Walker Minz, Professor and Head, Immunopathology (Co-PI),
Dr BR Thapa, Professor, Gastroenterology, pediatric division (Co-PI),
Dr Veena Dhawan, Additional Professor, Experimental Medicine & Biotechnology (Co-Invest),
Dr Ravi Kanojia, Assistant Professor, Pediatric Surgery (Co-Invest)

Name of Participant: _______________________________________________

Title: Identification and characterization of target antigens on biliary epithelial cells and in blood in patients of biliary atresia (BA) using a proteomic approach.

You are invited to take part in this research study. The information in this document is meant to help you decide whether or not to take part. Please feel free to ask if you have any queries or concerns.

You are being asked to participate in this study being conducted in PGIMER, Chandigarh because you satisfy our eligibility criteria which are:

(1) Diagnosis of BA
(2) Age <12 years

You will be one of the 20 patients/10 control subjects we plan to recruit in this study. Once recruited, you will be assigned in to either of the disease or control group.

What is the purpose of research?

BA is life threatening and most common cause of obstructive jaundice in infancy with limited therapeutic options. The treatment hurdles are persisting because the exact etiology of the disease is unclear. To improve treatment results, elucidation of the underlying mechanism of destruction of biliary tract leading to BA is crucial. The identification and characterization of unknown target antigens may be of great help in understanding the pathogenesis of the disease. The study may also be useful for the development of an early diagnostic biomarker and in designing therapies targeting that antigen.

Participant’s initials……………..
**The study design**

About 3 ml venous blood will be drawn both from patients just before surgery and control subjects. The epithelial cells from the tissue provided after surgery will be isolated and cultured. Proteins from cells and serum obtained from blood will be extracted and estimated followed by 1 and 2 dimension gel electrophoresis. The protein spots of interest will then be subjected to MALDI-TOF followed by their identification and characterization using online database. The target protein(s) identified then will be introduced in to mice for induction of experimental BA in them which will further evaluate the role of target proteins as an etiological factor.

**Study Procedures**

Once you are enrolled in the study, you will be required to follow the instructions:

You will be told about your visit schedules and you will have to report to the hospital (study site).

You are not allowed to take any medications other than the ones prescribed by your treating physician. If you need to take some treatment (drug/physiotherapy/other), you must consult your treating physician before taking that treatment.

At each visit, the study physician will examine you. Some [blood / urine / other] tests will be carried out at each visit. These tests are essential to monitor your condition, and to assess the safety and efficacy of the treatment given to you.

In addition, if you notice any physical or mental change(s), you must contact the persons listed at the end of the document.

You may have to come to the hospital (study site) for examination and investigations apart from your scheduled visits, if required.

**Possible risks to you**

No significant risk, except the minor risks, is associated with collection of blood by venupuncture.

**Possible benefits to you**

You are not expected to get any benefit from being on this research study, other than the treatment benefit and free investigations/tests.

**Compensation**

You are not expected to get any benefit from being on this research study, other than the treatment benefit and free investigations/tests.

Participant’s initials……………..
Possible benefits to other people
The results of the research may provide benefits to the society in terms of advancement of medical knowledge and/or therapeutic benefit to future patients.

The alternatives you have
If you do not wish to participate, you have the alternative of getting the standard treatment for your condition.

Cost to the participant
You will not be required to pay for the medications or lab tests required for the purpose of the study.
You will not be paid for your traveling expenses / you will not be paid to participate in this research study. In case of any adverse event occurring due to the study medications, you will be provided free treatment at our Institute and proper referral if necessary.

Who is paying for this research?
The DBT is the sponsor of the study and is paying for the research. [PGIMER receives money from the sponsor to conduct this study. The investigator or any of his/her team members does not receive any direct payment from the sponsor].

What should you do in case of injury or a medical problem during this research study?
Your safety is the prime concern of the research. If you are injured or have a medical problem as a result of being in this study, you should contact one of the people listed at the end of the consent form. You will be provided the required care/treatment.

Confidentiality of the information obtained from you
You have the right to confidentiality regarding the privacy of your medical information (personal details, results of physical examinations, investigations, and your medical history). By signing this document, you will be allowing the research team investigators, other study personnel, sponsors, institutional ethics committee and any person or agency required by law like the Drug Controller General of India to view your data, if required.
The results of clinical tests and therapy performed as part of this research may be included in your medical record. The information from this study, if published in scientific journals or presented at scientific meetings, will not reveal your identity.

Participant’s initials…………….
How will your decision to not participate in the study affect you?
Your decision not to participate in this research study will not affect your medical care or your relationship with the investigator or the institution. Your doctor will still take care of you and you will not lose any benefits to which you are entitled.

Can you decide to stop participating in the study once you start?
The participation in this research is purely voluntary and you have the right to withdraw from this study at any time during the course of the study without giving any reasons. However, it is advisable that you talk to the research team prior to stopping the treatment. You may be advised about how best to stop the treatment safely. If you withdraw, you may be asked to undergo some additional tests to which you may or may not agree. Though advisable that you give the investigators the reason for withdrawing, it is not mandatory.

Can the investigator take you off the study?
You may be taken off the study without your consent if you do not follow instructions of the investigators or the research team or if the investigator thinks that further participation may cause you harm.

Right to new information
If the research team gets any new information during this research study that may affect your decision to continue participating in the study, or may raise some doubts, you will be told about that information.

Participant’s initials…………..
Contact persons
For further information / questions, you can contact us at the following address:

Principal Investigator:
Dr. Yashwant Kumar
Dept. of Immunopathology, PGIMER, Chandigarh
Ph: +91722755191; Fax: +91722744401

Co-Principal Investigator
Prof B R Thapa,
Dept. of Pediatric Gastroenterology, PGIMER, Chandigarh
Ph: +91722756607; Fax: +91722744401

In case of conflicts, you can contact the chairperson (convener) of our Institutional ethics committee at the following address:
Prof. Dheeraj Gupta,
Convener/Member, Institutional Ethics Committee
Dept. of Pulmonary Medicine, PGIMER, Chandigarh

Participant’s initials……………..
Patient consent form

Title of the study: Identification and characterization of target antigens on biliary epithelial cells and in blood in patients of biliary atresia by proteomic approach.
Dr Yashwant Kumar, Assistant Professor, Immunopathology (PI),
Dr Ranjana Walker Minz, Professor and Head, Immunopathology (Co-PI),
Dr BR Thapa, Professor, Pediatric Gastroenterology (Co-PI),
Dr Veena Dhawan, Additional Professor, Experimental Medicine & Biotechnology (Co-Invest).
Dr Ravi Kanojia, Assistant Professor, Pediatric Surgery (Co-Invest)

Name of the Institution: Postgraduate Institute of Medical Education and Research, Chandigarh

Name and address of the sponsoring (funding) agency (ies): DBT, New Delhi

Documentation of the informed consent

I, have read the information in this form (or it has been read to me). I was free to ask any questions and they have been answered. I am over 18 years of age and, exercising my free power of choice, hereby give my consent to be included as a participant in “Identification and characterization of target antigens on biliary epithelial cells and in blood in patients of biliary atresia by proteomic approach”.

(1) I have read and understood this consent form and the information provided to me.
(2) I have had the consent document explained to me.
(3) I have been explained about the nature of the study.
(4) My rights and responsibilities have been explained to me by the investigator.
(5) I have informed the investigator of all the treatments I am taking or have taken in the past …… months including any desi (alternative) treatments.
(6) I hereby give permission to the investigators to release the information obtained from me as result of participation in this study to the sponsors, regulatory authorities, Government agencies, and ethics committee. I understand that they may inspect my original records.
(7) My identity will be kept confidential if my data are publicly presented.
(8) I have had my questions answered to my satisfaction.
(9) I have decided to be in the research study.

Participant’s initials…………….
I am aware, that if I have any questions during this study, I should contact at one of the addresses listed above. By signing this consent form, I attest that the information given in this document. I will be given a copy of this consent document.

**For children being enrolled in research**

Whether child's assent was asked: Yes No (Tick one)

[If the answer to the above question is Yes, write the following phrase:
You agree with the manner in which assent was asked for from your child and given by your child. You agree to have your child take part in this study.]

[If answer to the above question is No, give reason(s):]

Although your child did not or could not give his or her assent, you agree to your child's participation in this study.

Name and signature / thumb impression of the participant's parent(s) (or legal representative):

___________________________ (Name) _____________________________
(Signature)

___________________________ (Name) _____________________________
(Signature)

Date: __________________ Time:______________

Name and signature of impartial witness (required if parents of participant child illiterate):

___________________________ (Name) _____________________________
(Signature)

Date: __________________ Time:______________

Address and contact number of the impartial witness: ______________________

Name and signature of the Investigator or his representative obtaining consent:

___________________________ (Name) _____________________________
(Signature)

___________________________ (Date)

Participant's initials.............
Investigator Certificate

I certify that all the elements including the nature, purpose and possible risks of the above study as described in this consent document have been fully explained to the subject. In my judgment, the participant possesses the legal capacity to give informed consent to participate in this research and is voluntarily and knowingly giving informed consent to participate.

Signature of the Investigator: ________________ Dated __________
Name of the Investigator: ___________________

Participant’s initials……………..
### General information

<table>
<thead>
<tr>
<th>Name</th>
<th>Age</th>
<th>Sex</th>
<th>CR No</th>
<th>Address</th>
<th>Ph No.</th>
<th>Mobile</th>
<th>Landline</th>
<th>Date of admission</th>
<th>Date of surgery</th>
</tr>
</thead>
</table>

### History of illness

**Month & year of onset of illness**

**Duration of presenting symptoms**

**Presenting symptoms**

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
<th>Symptom/Findings</th>
<th>Month of onset</th>
<th>Duration</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>☐</td>
<td>☐</td>
<td>Jaundice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐</td>
<td>☐</td>
<td>Acholic stools</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐</td>
<td>☐</td>
<td>Dark urine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐</td>
<td>☐</td>
<td>Hepatomegaly</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐</td>
<td>☐</td>
<td>Splenomegaly</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐</td>
<td>☐</td>
<td>Slow growth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐</td>
<td>☐</td>
<td>Weight loss</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>☐</td>
<td>☐</td>
<td>Associated anomaly, if any</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Radiology/Surgery

- USG abdomen
- Mebrofenin hepato-biliary scan
- Per-operative cholangiogram
- Per-operative scrapings
- Liver biopsy

### Lab investigations

<table>
<thead>
<tr>
<th>Date</th>
<th>Sr Bil (total)</th>
<th>direct</th>
<th>indirect</th>
<th>SGOT</th>
<th>SGPT</th>
<th>GGT</th>
<th>Alk phos</th>
</tr>
</thead>
</table>

### Notes:

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**Post Graduate Institute of Medical Education & Research, Chandigarh, India**

**Patient performa**