BMJ Open Protocol for a diagnostic accuracy study to develop diagnosis algorithm for biliary atresia using MMP-7 (DIABA-7 study): a study recruiting from Chinese Biliary Atresia Collaborative Network

Jingying Jiang,¹ Jiale Deng,² Gong Chen,¹ Rui Dong,¹ Song Sun,¹ Min Du,¹ Dake Yang,² Zhuo Fang,² Shan Zheng ,¹ Chinese Biliary Atresia Collaborative Network

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JJ and JD contributed equally.

JJ and JD are joint first authors.

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For numbered affiliations see end of article.

Correspondence to

Dr Shan Zheng; szheng@shmu.edu.cn and Dr Zhuo Fang; fang_zhuo@wuxidiagnostics.com

ABSTRACT

Introduction Biliary atresia is a severe liver disease in neonates, and the prognosis partially depends on the age at which infants undergo the Kasai procedure. Matrix metalloproteinase-7 (MMP-7) was confirmed to have significant value in the diagnosis of biliary atresia. However, so far, the reference range and its cut-off value for diagnosing biliary atresia have not been established yet.

Methods and analysis Diagnosis Algorithm for Biliary Atresia (DIABA-7) is a prospective diagnostic test. Cholestatic infants and normal controls within 150 days of age are recruiting from the Chinese Biliary Atresia Collaborative Network. The serum samples and dried blood spot (DBS) samples are obtained to detect MMP-7 concentrations using an ELISA kit. The reference standard is the intraoperative exploration and subsequent histological examination of liver biopsies. Lambda-Mu-Sigma (LMS) method is used to calculate the normal range of serum MMP-7 of each age group. Receiver operating characteristics (ROC) curves are constructed to calculate the best cut-off point and area under the curve for the index test. The sensitivity, specificity, positive predictive value and negative predictive value are used to show the diagnostic accuracy. Pearson correlation coefficient test is applied to assess the correlation of serum MMP-7 and DBS MMP-7.

Ethics and dissemination This study was reviewed and approved by the Ethics Committee of Children's Hospital of Fudan University (Number 2020–296). Dissemination will be guided by investigators and patients. The aim is to publish the study results in a high-quality peer-reviewed journal and present the findings at international academic meetings.

Trial registration number ChiCTR2000032983.

INTRODUCTION

Biliary atresia (BA) is a severe liver disease in neonates, characterised by fibroinflammatory destruction of bile ducts.¹ Kasai portoenterostomy has been the consensual strategy

Strengths and limitations of this study

- This is a multicenter study in China recruiting 1200 consecutive cholestatic infants and 154 normal controls, which will be one of the largest diagnostic tests for biliary atresia (BA).
- The reference range and cut-off value for diagnosing BA of MMP-7 will be established so that to further standardise and popularise its clinical application.
- Diagnostic model based on serum biomarkers together with other parameters will help in the precise diagnosis for BA.
- External validation of the diagnostic cut-off value will be needed in the setting and population that the diagnosis algorithm will be used.

for BA since 1959.^{2,3} The age at which infants with BA undergo the Kasai procedure is a good clinical predictor of outcome, for those who undergo surgery within 60–90 days of age achieve a better prognosis.^{4–7} However, prompt treatment relies on early diagnosis. So far, it remains difficult to achieve perfect accuracy with either liver function test or grayscale ultrasound, which are most widely used in clinical practice.⁸

Matrix metalloproteinase-7 (MMP-7) plays an important role in extracellular matrix remodelling, which is closely related to the progression of liver fibrosis.^{9,10} In 2017, it was first reported by Bezerra *et al* that MMP-7 had a promising diagnostic value for BA, with an area under the curve (AUC) of 0.97, and the sensitivity and specificity were 97% and 91%, respectively.¹¹ In 2018, Yang *et al* from the same group further conducted a clinical diagnostic test and found that the AUC for diagnosing BA was 0.99 at the cut-off value of 52.85 ng/mL, and the sensitivity, specificity and positive

predictive value (PPV) were 98.67%, 95% and 98.28%, respectively.¹² Afterwards, groups from Taiwan and Shanghai also verified the diagnostic accuracy of serum MMP-7, and the sensitivity was 97.3% and 95.19%, and the specificity was 83.2% and 93.07%, respectively.^{13,14} However, the cut-off value varied, 10.37 ng/mL for the study from Shanghai, while only 1.43 ng/mL for that from Taiwan.

All the studies mentioned above highly confirmed the significant value of serum MMP-7 in the diagnosis of BA, whereas the differences in the cut-off value indicated that further research is warranted before its clinical application, to standardise the measurement of serum MMP-7 and confirm the reference range and also its cut-off value for diagnosing BA.

METHODS AND ANALYSIS

Study design

Diagnosis Algorithm for Biliary Atresia (DIABA-7) is a prospective diagnostic test. A study protocol for recruitment plan and detailed data collection method was designed and related medical professionals were trained before the study's start date.

Primary objective

To clarify the normal reference range of serum MMP-7 for each month age within 150 days after birth and the diagnostic cut-off value in diagnosing BA.

Secondary objectives

1. To analyse the value of serum MMP-7 in the differential diagnosis of BA in each age group within 150 days, especially in the neonatal group.
2. To develop a convenient and simple-operated method using dried blood spot (DBS) to measure MMP-7 concentration, which can be delivered at room temperature, and to explore its feasibility in screening and diagnosing BA.
3. To establish an accurate multivariate diagnostic model of BA using the deep machine learning model based on serum biomarkers together with other parameters.

Setting

DIABA-7 is recruiting from Chinese Biliary Atresia Collaborative Network, including paediatric surgery or gastroenterology outpatient clinics and inpatient departments in the tertiary hospitals in China including Children's Hospital of Fudan University, Children's Hospital of Fudan University Xiamen Branch, HaiNan Children's Hospital, Children's Hospital of Shanxi, Children's Hospital of Nanjing Medical University, Shengjing Hospital of China Medical University, Hunan Children's Hospital, etc. The multicentre recruitment will help to achieve the sample goal within the timeline and support the generalisability of the test.

PARTICIPANT SELECTION

Inclusion criteria

1. Cholestatic patients
 - A. Age: ≤ 150 days, no gender limit.

- B. Diagnostic criteria for cholestatic jaundice: serum total bilirubin $< 85 \mu\text{mol/L}$, but serum direct bilirubin $\geq 17 \mu\text{mol/L}$ or serum total bilirubin $\geq 85 \mu\text{mol/L}$ but direct bilirubin accounts for more than 20%.
- C. Gestational age ≥ 28 weeks, birth weight ≥ 1000 g.
- D. Sign the informed consent form.

2. Normal controls

- A. Age: ≤ 150 days old, no gender limit.
- B. Gestational age ≥ 28 weeks, birth weight ≥ 1000 g.
- C. No manifestations of cholestasis or other known hepatobiliary diseases.
- D. Sign the informed consent form.

Exclusion criteria

- A. A history of severe hypoxia at birth, Apgar score < 5 points.
- B. A history of allergic diseases, inflammatory bowel disease and infectious diseases.
- C. Complicated with other serious congenital malformations and immune deficiencies such as neurological, cardiovascular, pulmonary, endocrine and renal disorders that would interfere with the conduct and results of the study.

INDEX TEST

Sample collection

Blood samples of the cholestatic patients are obtained along with patients' routine laboratory tests at the outpatient clinics or at the admission to the inpatient unit.

Blood samples of the normal controls are obtained along with patients' routine laboratory tests at the admission to the inpatient unit from patients admitted for reasons other than hepatobiliary diseases.

The blood sample is centrifuged at 3000 rpm for 10 min to get the serum, and then the serum samples are stored at -80°C before measurement.

At the same time, a volume of $10 \mu\text{L}$ blood sample is obtained from the heel tip to a DBS patented by WuXi Diagnostics.¹⁵ After fully drying, it is stored at -20°C before measurement.

Serum MMP-7 concentration measurement

Serum MMP-7 concentration is measured using an enzyme-linked immunosorbent assay (ELISA, R&D, DMP700 and WuXi Diagnostics' Self-developed ELISA kit). Considering the measurement range of the kit, the serum samples of cholestatic patients are diluted 10 to 30 times. All the measurements are performed by WuXi Diagnostics who are blinded to other test results. Each sample is provided with three technical replicates in each assay, and the mean is recorded.

MMP-7 concentration measurement for DBS

Add $150 \mu\text{L}$ Calibrator Diluent (RD6-28) of the ELISA kit (R&D, DMP700) into the DBS tower, and then soak, shake (Thermo-shaker BE-9008) for 1 hour. Thereafter, $50 \mu\text{L}$ of the solvent extraction is loaded to detect the concentration of MMP7 on DBS using enzyme-linked

immunosorbent assay (ELISA, R&D, DMP700). Each sample is provided with two technical replicates, and the mean is recorded.

Serum IL-33 concentration measurement

Serum IL-33 concentration is measured using enzyme-linked immunosorbent assay (ELISA, R&D, D3300B). Each sample is provided with three technical replicates, and the mean is recorded.

Serum bile acid profile measurement

A liquid chromatography-tandem mass spectrometry system (self-developed kit by WuXi Diagnostics) is used to detect the concentration of each bile acid in human serum including cholic acid (CA), glycocholic acid, taurocholic acid, chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid, taurochenodeoxycholic acid, deoxycholic acid, glycodeoxycholic acid, taurodeoxycholic acid, ursodeoxycholic acid (UDCA), glycoursoxycholic acid, lithocholic acid, glycolithocholic acid, tauroolithocholic acid, taurohyocholic acid, hyodeoxycholic acid (HDCA), glycohyodeoxycholic acid, taurohyodeoxycholic acid, dehydrocholic acid.

Reference test

Intraoperative exploration and subsequent histological examination of liver biopsies are used to confirm the diagnosis of BA. If the gallbladders of some patients are seen to be completely atrophic, and the injection of contrast is hard to achieve, the baby will be diagnosed as BA at once. Otherwise, a cholangiography will be performed to further evaluate the anatomy of the intrahepatic bile ducts before a final diagnosis is made. Non-BA patients are confirmed by intraoperative cholangiography showing a patent biliary tree, percutaneous transhepatic biopsy excluding BA, genetic tests showing certain gene mutation or alleviation without surgical intervention during follow-up for at least 3 months. All the cases will be reviewed by the experts from the Chinese Biliary Atresia Collaborative Network before the final diagnosis is made.

Study flow

The study flow is depicted in [figure 1](#). Infants who meet the eligibility criteria will be consecutively recruited. After informed consent has been taken, all participants undergo the index tests and the reference test. All the index tests are performed by investigators from WuXi Diagnostics who are blinded to other test results. The reference test is performed afterward by the investigators (experts in paediatric surgery or gastroenterology) of each hospital. Though the investigators of each hospital have access to the results of index tests before making the final diagnosis, the biases are limited since the reference standards are definite and objective. Following that, information is extracted from the medical record including the demographic parameters, routine liver function test, sonographic features, index tests, reference standard (diagnosis of BA or Non-BA) and outcome after

Figure 1 Flowchart of the study patient. MMP-7, matrix metalloproteinase-7.

treatment. A summary of the data variables collected in the DIABA-7 study is presented in [table 1](#).

Data management

Data are collected at the time of measurement and from the medical record. Several steps have been taken to help ensure high-quality data collection. First, all DIABA-7 study investigators undergo standardised training before starting the study, including the sample collecting and storing, case report form filling. Besides, the electronic input is consistent with the content of the paper form. The data entry interface is established through Microsoft Access 2010 and uploaded to a central server on a weekly basis. Paper forms will also be available on the field as a backup.

Sample size

1. To clarify the normal reference range of serum MMP-7 and the diagnostic cut-off value in diagnosing BA, we set up the specificity of the test for distinguishing normal for case is 90%, using the formula: $n = \frac{[Z_{1-\alpha/2}]^2 \hat{p}(1-\hat{p})}{d^2}$, where $\hat{P}=0.9, \alpha=0.05, d=0.05. n=139$.
2. To establish the correlation between serum MMP-7 and DBS MMP-7, 150 cholestatic participants will be needed with a two-tailed α of 0.05 and β of 0.1, for achieving correlation coefficient r of 0.85.
3. To prove the efficiency of BA artificial intelligence (AI) multivariate diagnostic model is better than the single biomarker, 581 samples will be needed for each group, by comparing the AUC. Due to our group's preliminary results and other literature, the hypothesis is:
 $H_0: AUC_{AI} = AUC_{singlebiomarker}$
 $H_1: AUC_{AI} > AUC_{singlebiomarker}$
 where $AUC_{AI}=0.956, AUC_{singlebiomarker}=0.92$.
 Due to the statistical estimating above, the largest sample size request is 545 sample each group.

Continued

Patient ID: Number_____	
Triangular cord sign	← Yes, _____ (size) ← No
Gallbladder	← Yes, _____ (size) ← No
Common bile duct	← Yes, _____ (diameter) ← No
Hepatic artery	_____ (diameter)
Liver stiffness measurement	
Final diagnosis	
Surgical exploration	
Liver biopsy	
Gene test	
Other	

The least absolute shrinkage and selection operator regression method will be used to determine the optimal covariates to differentiate BA from non-BA. The patients will be divided into training set (70% data) and validating set (30% data) by random sampling function in R

software. The covariates picked will be used to build the multivariable logistic regression model. The accuracy of the regression model will be assessed by the discrimination ability in the training set and the validating set.

Statistically significant difference was defined as a *p* value less than 0.05. All data analyses are performed using R software (3.6.3, Vienna Australia).

Patient and public involvement

Patients and their guardians are not involved in the study design and are not consulted to develop patient-relevant diagnoses and outcomes or interpret the results. Patients are not invited to contribute to the writing or editing of this document for readability or accuracy. We intend to disseminate the main results to the families of the trial participants and will pursue patient and public involvement in the development of an appropriate method to disseminate this information.

ETHICS AND DISSEMINATION

Ethics

This study was reviewed and approved by the Ethics Committee of Children's Hospital of Fudan University (number 2020-296). The study will be performed in compliance with the Declaration of Helsinki and other relevant regulations.

The investigator has the responsibility to comprehensively introduce the purpose, procedures and possible risks of this study. An informed consent form must be given to each subject and be signed before recruitment.

The personal information will be kept confidential. Similarly, deidentified data are used for statistical evaluation. Only the investigators can identify the subject's name and other personal information by initials.

Disseminations

Dissemination will be guided by investigators and patients. The aim is to publish the study results in a high-quality peer-reviewed journal and present the findings at international academic meetings.

DISCUSSION

Experts from all over the world have made great contributions to exploring early and non-invasive diagnostic methods of BA during the past decades.

Stool colour cards can help identify the alcoholic stool in the early neonatal period, and the sensitivity for screening BA is around 76%–90% and the specificity is nearly 100%.^{16–20} In 2016, we launched the mobile phone application of stool colour card to simply and effectively differentiate abnormal stool from a normal one. Subsequently, a nomogram model based on five variables (gender, weight, direct bilirubin, alkaline phosphatase, glutamyltranspeptidase) was established based on big data with the sensitivity and specificity of 85.7%

and 80.3%, respectively,²¹ which helps the general practitioner to predict the risk of BA in cholestatic infants.

The age of Kasai operation in children with BA before and after stool colour card screening and nomogram-assisted diagnosis (2014–2016 vs 2017–2019) in our hospital was statistically analysed. The results showed that the age of Kasai operation in the second 3 years (62.68±19.60d) was earlier than that in the first 3 years (66.68 days ± 24.89 days) (*p* > 0.05).

Considering the convenience and accuracy of MMP-7 in diagnosing BA, a standardised diagnosis algorithm should be established to further improve the age of Kasai operation.

Study process

Seven tertiary hospitals in China are now open for recruitment. These centres are Children's Hospital of Fudan University, Children's Hospital of Fudan University Xiamen Branch, HaiNan Children's Hospital, Children's Hospital of Shanxi, Children's Hospital of Nanjing Medical University, Shengjing Hospital of China Medical University, Hunan Children's Hospital. The first participant was recruited in August 2020 and the study is due to finish recruiting in August 2022. We are currently in the subject recruitment. Other clinical centres for BA are still welcome to join in the Chinese Biliary Atresia Collaborative Network and the subject recruitment.

Author affiliations

¹Department of Pediatric Surgery, Children's Hospital of Fudan University, Shanghai, China

²Department of Data & Analytics, WuXi Diagnostics, Shanghai, China

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Collaborators Children's Hospital of Fudan University, Children's Hospital of Fudan University Xiamen Branch, HaiNan Children's Hospital, Children's Hospital of Shanxi, Children's Hospital of Nanjing Medical University, Shengjing Hospital of China Medical University, Hunan Children's Hospital, Children's Hospital of Xuzhou, Affiliated Hospital of Zunyi Medical University, Chengdu Women's and Children's Central Hospital

Contributors SZ, ZF, JJ, JD conceptualised and designed the study, drafted the initial manuscript and reviewed and revised the manuscript. JJ, JD, DY, MD designed the standard questionnaire and the data collection instruments, collected data and reviewed and revised the manuscript. SZ, ZF, GC, RD, SS conceptualised and designed the study, coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Consent obtained from parent(s)/guardian(s).

Provenance and peer review Not commissioned; externally peer reviewed.

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ORCID iD

Shan Zheng <http://orcid.org/0000-0002-9712-4573>

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