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Blood and Genomics

2023, 7(2): 77−85

B & G

Review Article

Clinical practice of ABO blood group molecular analysis: Chinese expert consensus

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Conflict of interests: The authors declared no conflict of interests.

https://doi.org/10.46701/BG.2023022023023

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ABSTRACT

The ABO blood group, based on molecular biological detection technology, has the advantages of simple operation, high sensitivity, and standardized result interpretation, and is not affected by sample immunological characteristics. However, clinically, performance verification, clinical application scope, quality management, abnormal result processing, and other issues associated with the ABO blood group molecular detection technology are relatively complex, and there is a lack of unified norms and standards. Therefore, from the perspective of the whole process of ABO molecular biology detection, this study aims to provide standardized opinions on important links affecting the detection results, common problems encountered in the detection process, and the assessment and treatment of abnormal results. Finally, a Chinese expert consensus on molecular biological technology based on genotyping and sequencing detection was put forward, which standardizes the detection process, improves the accuracy of results, and promotes the development of technology and broader clinical application.

Keywords: ABO blood group, molecular detection technology, genotyping detection, sequencing detection, ABO allele

INTRODUCTION

Molecular detection of erythorbic ABO blood group can be used as an auxiliary diagnosis for clinical samples due to immunological or pathological limitations, and can effectively distinguish subtypes from disease-related discrepancies[1–2]. The commonly used molecular red blood cell (RBC) blood group detection platforms in China include the Taqman probe and SYBR real-time polymerase chain reaction (PCR), Taqman probe PCR, electrophoresis for genotyping, and Sanger method sequencing-based testing (SBT) for mutation point analysis. Genotyping offers the advantages of simple operation, short time consumption, and high sensitivity, and can be used for the rapid screening of samples. However, it relies on SBT for ABO subtype analysis[3]. Molecular detection technology has recently been widely applied in blood group detection[4]. The standardization of detection is of great significance in improving RBC detection levels and promoting individualized diagnosis and treatment.

This consensus was jointly initiated and formulated by clinical transfusion and pathology experts with rich theoretical and experimental experience, aiming to provide guidance for the practice of ABO blood group testing technology. Included are important parts of clinical practice, such as sample selection, preprocessing, detection process, performance verification, quality management, ABO single nucleotide polymorphisms, clinical application, processing of common problems and abnormal results, and detection limitations. ABO blood group testing technology can be a reference for blood transfusion departments, laboratories, and commercial testing institutions to carry out ABO blood group molecular testing.

Based on clinical practice data and characteristics of the Chinese population, the recommendations are divided into three levels: strongly recommended (measures with sufficient evidence and consensus reached by the experts, which are preferentially recommended in the settings with sufficient testing conditions), recommended (measures with sufficient evidence and consensus reached by the experts, which are recommended based on actual testing conditions), and not recommended.

SAMPLE PRE-PROCESSING

Sample acquisition, transportation, and storage

Peripheral blood samples are generally collected using ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes, which can be transported and stored at room temperature. Complete plasma separation should be carried out within 2 hours after blood collection. The separated plasma can be temporarily stored at −20 ℃ (≤1 week) or −80 ℃ (≤1 month). The detection should be completed within 1 week. The remaining biological samples and nucleic acids should be classified and stored to trace the causes when problems arise in subsequent testing[5].

Sample source and nucleic acid extraction

ABO blood group molecular detection is mainly based on anticoagulant whole blood, according to the sample source and requirements set out in the instructions of whole blood nucleic acid extraction reagents. Recent transfusion history does not affect molecular biological testing in most cases[6–7].

Commonly used nucleic acid extraction methods in clinic include column and magnetic bead methods. Laboratories can select appropriate extraction methods and kits based on their actual conditions. The commercialized kits registered by the National Medical Products Administration (NMPA) are recommended.

If laboratory-developed or other nucleic acid extraction reagents are used, performance verification should be carried out to meet the experimental application requirements. The qualified DNA absorbance (A) 260/280 ratio should be between 1.7–2.0, and the amount of nucleic acid extracted from a whole blood sample in a single time should be ≥ 800 ng[8–9].

Sample limitations

The molecular test results of patients with chimera may only present the dominant group of the stem cell line, or may not be comprehensible. The presence of free DNA in pregnant women with twins cannot be accurately used to detect individual fetal results.

Several sample statuses can not effectively carry out nucleic acid extraction or donor's genome interference, including recent massive blood transfusion, low white blood cell count or severe immunosuppression. Hence, the use of the oral mucosa or other patient tissues for nucleic acid extraction may be considered[10–11].

DETECTION PROCESS AND RESULT ANALYSIS

Molecular biological detection

The commercialized kits authorized by NMPA are strongly recommended for testing. The samples should be sent to a fully qualified commercial laboratory. Moreover, laboratories should develop their detection capabilities to meet the requirements described in the ABO molecular detection section and achieve detection consistency in a certain number of samples with NMPA registered kits or commercial detection results. Besides detection methods, laboratories should establish a corresponding standard operating procedure (SOP) for sample transmission and recording, information management, experimental operation, and report issuance.

PCR system

While preparing the PCR system, the following points should be noted: (1) The nucleic acid should be diluted to the specified concentration range following the manufacturer's instructions. Inappropriate nucleic acid concentration may lead to false positive and false negative results. (2) Before adding samples, the detection reagent should be completely melted, thoroughly shaken, and mixed, and instantaneous centrifugation should be used as a backup. The thawing reagent can be temporarily stored at 2–8 ℃ (no more than four times is recommended) to avoid repeated freezing and thawing, and should be used according to the manual's instructions. (3) During sample loading, repeated blowing of the pipette must be avoided to reduce the possibility of air bubbles and aerosol pollution, and the filter tip can avoid aerosol pollution. (4) After completing sample loading, the PCR tube should be centrifuged instantaneously, dispersed, and symmetrically placed in the PCR instrument, and the amplification procedure should follow the SOP[12]. (5) After PCR amplification, whether the internal reference result/negative quality

control and positive/weak positive quality control are under control should be confirmed, and then the sample test results can be analyzed. If any test well result is out of control, conducting the testing again is necessary[13–14].

Issuance of test reports

Each medical institution/commercial laboratory should set up a standardized result report template according to the actual situation, and the transfusion physician and molecular testing personnel should cooperate to complete the molecularly pathological diagnosis report promptly and accurately to provide a reference for diagnosis and treatment.

The genetic testing report includes but is not limited to the following aspects: (1) patient and sample information, including patient's name, sex, age, outpatient or residence number, sample's source, number, type, and receiving time, clinical diagnosis, and nucleic acid quality control information; (2) detection items and methods, including instruments and reagent performance (such as sensitivity, specificity, limitations, detection scope, and mutation site); (3) results, including detection of gene variant sites and analysis report. The nomenclature following by the International Society for Blood Transfusion (ISBT) is recommended for gene variant sites. Moreover, it is recommended to express the two allele combinations and list the effective analysis fragments or gene variation sites. If other opinions or suggestions are available, they can be noted in the results; (4) description of limitations: adding remarks after the test results to explain the scope of responsibility and limitations of the test. For the polygenic blood group regulation system, any possible limitations of detected fragments and undetected genes should be particularly clear. If possible, serological and molecular biological test results can be simultaneously reported; (5) the report should be reviewed, signed by an authorized signatory with molecular diagnosis qualifications, and issued when applicable[15–17].

ABO MOLECULAR DETECTION

Reference and subtype allele database

The A1.01 allele is the only template for ABO blood group detection, and other common B or O blood group genes are also compared with the A1.01 gene. The subtype gene database can generally be divided into four secondary groups, including A subtype, B subtype, O subtype, and A-B hybrid gene. Subtype A establishes allele data of other subtypes according to serological characteristics based on the A1.01 allele. Subtype B and O establish a subtype database of B1.01 and 01.01 genes respectively after comparing different gene variations with A1.01. The A-B hybrid gene is mainly cis-AB and B (A) subtype, and the A1.01 gene is still used as the template. The results of direct molecular detection of the genome include the combined results of two alleles, and the report form needs to represent the combination of two alleles. If clarifying the haplotype of a specific mutation point is necessary, it is essential to perform pedigree analysis or clone sequencing confirmation[18].

The ABO subtype allele database is mainly based on the blood group system allele database updated on the ISBT website, and the allele information included in the database is also subject to serological testing[19]. However, the website lacks regional frequency information. The erythrogene website can be used as a supplement[20]. Additionally, the blood group antigen gene mutation database established from 1999 to 2018 contains the most abundant subtype alleles[21], although it has been stopped from updating new information.

The most common ABO allele genotypes in China include A1.01, A1.02, B1.01, O01.01, and O01.02. Owing to ethnic distribution, the A2.05 blood group should also be included (***Table 1***). Among 15 articles on A2 subtype molecular biology and gene frequency included in the CNKI, 203 cases were detected in A2.05, accounting for 67% of all A2 subtypes[22–23].

Table 1 **ABO common alleles in Chinese**

Alleles	Population distribution	Recommendation level
A1.01	12.00%	Strongly recommended
A1.02	11.00%	Strongly recommended
O. 01.01	32.00%	Strongly recommended
B. 01	21.00%	Strongly recommended
O. 01.02	24.00%	Strongly recommended
A205	1.02%	Recommended
A201	0.23%	Not recommended

Gene variation sites and specificity

Commercialized reagents or laboratory-developed testing can be used to develop and design primers according to the clinical demands for ABO detection. However, the specificity of the detection variant site or information shared with other subtypes must be clear. When enough sample population information is unavailable, the genotyping test should not present the allele results.

According to information on the public database, the base information of exons 1–7, intron 5, and intron

6 of the ABO gene fragment (alpha 1-3-n-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase) was analyzed to obtain the following characteristic mutation points (***Table 2***). This information provides guidelines for molecular biology detection, primer design, and detection limitations.

Clinical use and detection limitations

ABO genotyping can be used for the preliminary detection of subtype classification or the auxiliary assessment of ineffective serological testing due to physiological and pathological factors. However, ABO genotyping is not suitable for the confirmation of subtype alleles. If it is necessary to confirm the subtype allele, sequencing is the preferred tool.

ABO genotyping can assist in the assessment of traditional serological methods for ABO subtype identification, such as saliva or absorption-elution tests, and can obtain excellent diagnostic power. For example, the ABO genotyping detection method has the advantages of accuracy, rapidity, and convenient operation in patients with the para-Bombay blood group compared with the saliva or absorption-elution test[24–25]. Additionally, ABO genotyping can also be used as the primary screening and classification method for ABO subtype alleles and normal blood groups. In clinical disease application, when a serologic test is not able to determine blood groups in patients with autoantibodies or patients with tumors or blood diseases due to the weakening of the ABO antigen, genotyping can be used to preliminarily distinguish the ABO subtype and O allele[26–27]. The clinical diseases recommended for ABO molecular biology detection are presented in ***Table 3***.

Internal quality control or enterprise standard

The internal quality control products should meet the requirements in ***Table 4***. The internal quality control test should be performed when different batch numbers or reagents from different manufacturers/ methodologies are substituted. For the detected alleles, the inclusion of five alleles is strongly recommended: A1.01, A1.02, B.01, O01.01, O01.02, and A2.05. The enterprise standard should conform to the internal quality control standard, contain the test mutation site of commercial reagent, have positive and negative quality control results of the site, and meet the required expectations.

Detection limitations and multivariate analysis

ABO blood group antigen expression, ABO alleles, and the related promoters Runx1, GATA-1, c/ebp, and fucosyltransferase (FUT1 and FUT2), are the main molecular level factors regulating the generation of ABO antigens. The test's content should be explicit when issuing and interpreting the report, and the limits of the test results of undetected fragments should be described[28].

Aside from the molecular level regulation of ABO blood group antigens, physiological and pathological factors and special immunosuppressive therapy may affect the positive and negative results of ABO blood type. When interpreting the report, the patient's age, recent blood transfusion history, immunosuppressive therapy, and transplantation history should be comprehensively considered, especially for the chimeric blood group, where molecular biology can only effectively detect the dominant cell line.

GENERAL PROBLEMS AND SOLUTIONS

Genotyping

A risk of incorrectly loading samples in the electrophoresis genotyping process exists, which can be effectively avoided if the laboratory personnels are experienced and have the appropriate molecular biology operation qualifications. If each reaction well of the Taqman method is positive, it is most likely due to probe failure. The experiment should be repeated.

In the case of abnormal results or failure of any PCR reaction well, such as internal control failure, the experiment should be repeated, as interpretations cannot rely on incomplete experimental results.

When the genotyping results cannot reveal the expected alleles and results specified in the instructions, SBT should be performed.

Sequencing detection

Many overlapping peaks appear owing to the deletion or insertion mutation of a certain allele. All sequencing should be bidirectional, interpretation should be repeated as much as possible, and at least upstream and downstream 50 bps of exons should be effectively covered. The two-way interpretation results should be consistent to avoid overlapping peaks that cannot be interpreted or failure of effective mutation point detection.

DISCUSSION

The application of molecular biology technology for ABO blood group gene detection has the advantages of strong sensitivity and specificity, convenient operation, and closed tube detection.

Genotyping is suitable for gene point mutations and deletion mutations of known alleles (strongly

Table 2 **ABO SNPs of common alleles and subtypes alleles**

Exon/intron	Allele	A1.01 (Ref)	A1.02	A2.01	A2.05	B.01	O01.01	O01.02	Candidate SNPs for ABO genotyping
EX3	106	G	G	G	G	G	G	T	O subtype SNPs
EX4	188	G	G	G	G	G	G	A	O subtype SNPs
	189	C	C	C	C	C	C	T	O subtype SNPs
	190	G	G	G	G	G	G	G	O subtype SNPs
EX5	220	C	C	C	C	C	C	T	O subtype SNPs
Int5	103	C	C	C	C	C	C	Ins	O subtype SNPs
	306	C	C	C	C	C	C	T	O subtype SNPs
	336	G	G	G	G	G	A	G	O subtype SNPs
	450	C	C	C	C	C	C	A	O subtype SNPs
	527	G	G	G	G	G	A	A	A common SNPs*
	530	A	A	A	A	G	A	A	A common SNPs*
EX6	261	G	G	G	G	G	Del	Del	O common SNPs*
	297	A	A	A	A	G	A	G	B. O subtype SNPs
INT6	42	G	G	G	G	T	G	G	B common SNPs*
	89	T	T	T	T	T	T	A	O subtype SNPs
	163	T	T	T	T	C	T	C	B. O subtype SNPs
	179	C	C	C	C	T	C	C	B common SNPs*
	188	G	G	G	G	G	G	A	O subtype SNPs
	226	C	C	C	C	C	C	T	O01.02*
	235	C	C	C	C	C	C	G	O01.02*
	271	A	A	A	A	G	A	A	B common SNPs*
	280	C	C	C	C	T	C	C	B common SNPs*
	446	A	A	A	A	G	A	G	B. O subtype SNPs
	493	T	T	T	T	T	T	C	O subtype SNPs
	593	G	G	G	G	G	G	C	O subtype SNPs
	628	A	A	A	A	G	A	A	O subtype SNPs
	717	G	G	G	G	G	G	A	B subtype SNPs
	784	G	G	G	G	G	A	G	O01.01*
	786	A	A	A	A	G	A	G	B. O subtype SNPs
	891	A	A	A	A	G	A	G	B. O subtype SNPs
	901	G	G	G	G	A	G	G	B subtype SNPs
	950	A	A	A	A	G	A	G	B. O subtype SNPs
	1011	A	A	A	A	A	A	G	O subtype SNPs
	1013	G	G	G	G	G	G	A	O subtype SNPs
EX7	467	C	T	T	T	C	C	C	Several A subtype SNPs
	526	C	C	C	C	G	C	C	A2, B3, B (A) SNPs
	646	T	T	T	T	T	T	A	Aw, B3, O SNPs
	657	C	C	C	C	T	C	C	O subtype SNPs
	681	G	G	G	G	G	G	A	Aw, B3, O SNPs
	703	G	G	G	G	A	G	G	B3, O SNPs
	771	C	C	C	C	C	C	T	A2, O SNPs
	796	C	C	C	C	A	C	C	B (A), O SNPs
	803	G	G	G	G	C	G	G	Severe B subtype SNPs
	829	G	G	G	G	G	G	A	A2, Aw SNPs
	930	G	G	G	G	A	G	G	B (A), O SNPs
	1009	A	A	A	G	A	A	A	A2.05 SNPs*
	1061	C	C	Del	C	C	C	C	A2 and Aw SNPs

SNPs: single nucleotide polymorphisms; Ref: reference template; Int: intron; Ex: exon; *: the comon SNPs for A, B, O and specific SNPs for A2.05, O1.01, O1.02, which are potential SNPs for allele-specific primer design in Chinese.

Table 3 **Indications and recommendation levels for ABO molecular biological detection**

	Genotyping	Sequencing
Detection indications and recommendation levels	**Strongly recommended** *Immunological limitations* Direct Coomb test positive Auto-control positive *Subtype primary diagnosis*	**Strongly recommended** *Subtype primary diagnosis* When genotyping cannot explain serological results recommendation
	Recommended *Disease related weak ABO antigen* Lymphoma Leukemia Tumor or infection *Recent transfusion* *Transplant*	
	Not recommended *Prediction of hemolytic disease of newborn* *Chimera*	

Table 4 **Recommended rules for source of quality control products**

Rule description
• The real human genome sample or the whole cloned genome of the ABO gene (9q34.2).
• It is suggested to have a pedigree analysis or confirm the haplotype of the mutation point by cloning and sequencing.
• It is suggested to check the mutations by SBT (the sequencing effective fragment at least includes exons and 50 bps upstream and downstream).
• The molecular analysis should effectively explain the serological test results.
• The target detection sites of genotyping must be mutually verifiable with the results of SBT.
• The recommended source is included in the allele list in ***Table 1***.
• The source used for genotyping test must obtain positive and negative results.

recommended). Sequencing should be used to detect unknown mutation types or mutation sites outside the detection scope of the kit, including genes without clear hotspot mutations (strongly recommended). However, using genotyping to directly represent alleles does not have limitations in analyzing mutation sites beyond the detection points (not recommended).

A reasonable and feasible SOP should be developed according to the detection methodology and sample type, and the sample collection, transportation, receiving/rejection, storage, and pre-treatment processes should be specified in detail (strongly recommended). Before using any method for nucleic acid extraction from clinical samples, its efficiency and quality should be evaluated, and its performance should be verified (strongly recommended). Genotyping and sequencing should be performed using the kit registered/filed by NMPA or a detection platform developed by a certified commercial laboratory, and the detection SOP should be established following the manufacturer's instructions and existent laboratory conditions (strongly recommended). A standardized result report template should be set up, and the scope of responsibility and limitations of the test should be explained (strongly recommended). Laboratories should establish a perfect quality management system and carry out performance verification, internal quality control, and inter-laboratory quality assessment before performing new molecular biology testing to continuously improve quality (strongly recommended). In case of abnormal results in molecular biology, the experimental process should be checked according to the experimental records and SOP (strongly recommended). Measures should be taken to prevent, monitor, control, and eliminate pollution (strongly recommended). After excluding the problems of molecular detection experiments, the results should be interpreted according to the interpretation standards in the instructions. If necessary, the kit or other manufacturers' detection methods can be used for verification, or the samples can be sent to other qualified medical institutions' laboratories or commercial laboratories for detection and comparison (strongly recommended). For samples with no ABO subtype mutation detected in molecular biology, a comprehensive assessment of multi-dimensional factors such as the related promoter, fucosyltransferase gene, the patient's physiological and pathological status, immunosuppressive therapy, recent blood transfusion history, and transplantation

history should be considered to identify patients with rare mutation types with potential therapeutic benefits (recommended).

Acknowledgments

This expert consensus was supported by the grants from the National High Level Hospital Clinical Research Funding (No. BJ-2022-187) and the CAMS Innovation Fund for Medical Sciences (CIFMS) (No. 2021-I2M-1-060).

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Received 15 December 2023, Revised 21 December 2023, Accepted 22 December 2023