INTRODUCTION

Hereditary spherocytosis (HS) is a hereditary hemolytic anemia caused by the mutations in the genes encoding erythrocyte membrane proteins and is mostly inherited in an autosomal dominant manner.\textsuperscript{1-3} HS is classified into four types according to the severity of the disease: trait, mild, moderate, and severe. Commonly, there is heterogeneity of the clinical manifestations in patients.
with different HS types. Typical symptoms of HS include anemia, jaundice, and splenomegaly. However, patients with anemia such as thalassemia (THAL) and autoimmune hemolytic anemia (AIHA) also present with anemia, jaundice, and splenomegaly. Commonly used diagnostic tests for HS have no ideal sensitivity or specificity. Therefore, some HS patients are easily misdiagnosed or missed. Here, we propose a simple and practical diagnostic protocol for HS based on the results of existing experimental technologies and research, in an attempt to help clinicians improve their diagnosis of HS.

1.1 | HS is a common hereditary hemolytic anemia

HS has been reported worldwide with an incidence of about 1/2000 people in Northern Europe, and it is the most common hereditary hemolytic anemia in Japan. According to a report by Kutter, the incidence of HS is up to 1/150 in men and 1/800 in women, when advanced dual-beam laser technology was used for the screening. In China, there is still a lack of accurate HS epidemiological survey data, but related reports are common. Li et al. reported that HS accounted for 31.5% of 356 cases of hereditary hemolytic disease. Wang et al. found among 140 children with hemolytic anemia admitted to their hospital that 17.9% were diagnosed with HS.

1.2 | Ease of misdiagnosis or missed HS

In 2009, Wang et al. proposed the need to improve the diagnostic level of HS in response to its high incidence and difficult diagnosis. However, some reports on misdiagnosis or missed diagnosis of HS still exist in recent years. Misdiagnosis or missed diagnosis of HS is mainly due to: (a) the obviously heterogeneous clinical manifestations of HS; (b) the sensitivity or specificity of some diagnostic tests commonly used in the current HS diagnostic protocol, such as the mean corpuscular hemoglobin concentration (MCHC) detection, erythrocyte osmotic fragility test (OFT), peripheral red blood cell morphology examination, which are not ideal; and (c) there is insufficient knowledge of HS.

The clinical manifestations of HS (anemia, jaundice, and splenomegaly), and a common complication (cholelithiasis), are similar to those in patients with anemia such as THAL and AIHA. Therefore, it is difficult to distinguish HS from other types of anemia. For example, a male patient who had jaundice but no symptoms of anemia was misdiagnosed as having autoimmune hepatitis, while his 5-month-old son who had obvious symptoms of anemia and jaundice was misdiagnosed as having AIHA. Both were finally diagnosed with HS. Zhong et al. reported four cases of HS patients who had been treated in many hospitals due to "various degrees of anemia and jaundice," and had been misdiagnosed or informed of having an unknown diagnosis for a long time. Moreover, severe HS with typical symptoms can also be misdiagnosed and mistreated for a long time. Clinical misdiagnosis and mistreatment can both cause physical harm to the patient and increase the patient’s medical expenses.

The clinical manifestations and severity of HS are related to the types of membrane protein defects that occur. To date, the main membrane protein defects in HS involve ankyrin, α-spectrin, β-spectrin, band 3, and protein 4.2, which are encoded by ANK1, SPTA1, SPTB, SLC4A1, and EPB42 genes, respectively (Table 1). Mariani et al. found that band 3 protein defects accounted for about 54% of 300 European patients with HS. Park et al. found that ankyrin protein defects accounted for about 52% of HS patients in South Korea. Patients with α-spectrin protein defects have been reported to develop severe anemia, while patients with ankyrin, band 3, and protein 4.2 defects have mild to moderate anemia. In short, there are regional and inter-population differences in the types of membrane protein defects in HS patients, which also bring about difficulties in the diagnosis of HS.

The HS diagnostic protocol developed by Shen et al. proposed that if a patient has more microspherocytes (>10%) in the peripheral blood, with increased red blood cell permeability and fragility, and a positive family history, he/she, regardless of the symptoms, can be diagnosed with HS. The HS diagnostic guidelines developed by Bolton-Maggs et al. proposed that HS can be confirmed with no need to perform other tests when patients have a positive family history, typical clinical features, and abnormal laboratory test results (peripheral blood spherocytosis, MCHC increase, reticulocyte increase). However, the existing HS diagnosis protocols are usually difficult to be used by the clinicians in the following aspects:

1. When the patients’ parents have trait and mild HS with no obvious symptoms, it is easy to report this as a negative family history.
2. The laboratory tests, such as MCHC, OFT, and peripheral red blood cell morphology examinations, are not sufficiently sensitive or specific.

| Main defective membrane proteins | Encoding gene | Chromosome | Position | Mode of inheritance |
|----------------------------------|---------------|------------|----------|---------------------|
| Ankyrin                          | ANK1          | 8          | p11.2    | AD, AR              |
| α-Spectrin                       | SPTA1         | 1          | q22-23   | AR                  |
| β-Spectrin                       | SPTB          | 14         | q23-24.1 | AD                  |
| Band 3                           | SLC4A1        | 17         | q21-22   | AD                  |
| Protein 4.2                      | EPB42         | 15         | q15-q21  | AR                  |

Abbreviations: AD, autosomal dominant; AR, autosomal recessive.
3. Glucose-6-phosphate dehydrogenase (G6PD) deficiency, AIHA, and other diseases can be accompanied by peripheral blood spherocytes.\(^7,14\)
4. OFT can be positive for hereditary elliptocytosis (HE), AIHA, and other diseases.\(^9,24\)
5. The accuracy of the peripheral red blood cell morphology examination is related to the time of specimen placement and the level of inspectors.
6. Increased bilirubin level in the blood can lead to a false increase in MCHC values.\(^25\)

2 | LABORATORY TESTS FOR HS

2.1 | Routine blood test

Currently, the whole blood cell parameters used for HS diagnosis include hemoglobin (Hb) level, reticulocyte absolute count (Ret), MCHC, mean spheroid cell volume (MSCV), mean corpuscular volume (MCV), and mean reticulocyte volume (MRV). MCHC and MCV are indirect parameters measured by the blood cell analyzer, which are calculated according to the results of red blood cell count (RBC), Hb, and hematocrit (HCT). MCHC is calculated as Hb divided by HCT and MCV as HCT divided by RBC. MCV is a parameter directly measured by blood cell analyzer and can also be multiplied by RBC and MCV.\(^{24,25}\) After staining with new methylene blue, red blood cells are treated with an acidic and hypotonic solution to form deproteinized spherocytes. Spherocytes will be divided into mature erythrocytes and reticulocytes by a unique technique based on volume, conductivity, and light scattering optical detection. The average volume of all erythrocytes (both mature erythrocytes and reticulocytes) is defined as MSCV.\(^{26,27}\) MRV refers to the average volume of all reticulocytes and is also one of the reticulocyte parameters in the blood analyzer. MCHC, MCV, and MRV can be detected in the hematology analyzers produced by many companies, including Beckman Coulter, Simens, Horiba and Mindray. However, only the hematology analyzer produced by Beckman Coulter can report MSCV.\(^{28-30}\)

For the trait form of HS, Hb level and Ret (<3%) are normal; for mild HS, Hb level is 110–150 g/L and Ret is 3%–6%; for moderate HS, Hb level is 80–120 g/L and Ret >6%; and for severe HS, Hb level is 60–80 g/L, Ret >10%.\(^1,9\) We found that when MCHC >355 g/L is used as the threshold for diagnosing HS, the sensitivity and specificity are 41.07% and 94.47%, respectively;\(^31\) when MCHC ≥334.9 g/L is used, the sensitivity and specificity are 82.1% and 94.5%, respectively.\(^29\) When the blood bilirubin level increases, MCHC detection may be affected, with a false increase.\(^25\) Chiron et al.\(^24\) found that when MSCV <MCV is used as the threshold for diagnosing HS, the sensitivity and specificity are 100.0% and 93.3%, respectively. Therefore, we selected patients with HS, THAL, and healthy controls as research subjects, and found that when MSCV <MCV is used as the threshold for diagnosing HS, the sensitivity and specificity are 100% and 96.7%, respectively.\(^32\) Our further study also found that the measurement of MCV >MCV can be considered as an ideal index to quickly distinguish HS from THAL.\(^33\) We also compared HS, THAL, AIHA, and G6PD deficiency patients with healthy controls. It was found that MCV is a routine and specific screening index for HS, which is of great significance for the differential diagnosis of different types of hemolytic anemia. When MRV ≤95.77 fL is used as the threshold for diagnosing HS, the sensitivity and specificity are 86.80% and 91.20%, respectively.\(^34\) Nair et al.\(^35\) compared HS, AIHA, and healthy controls, and the results showed that when MCV - MSCV >10 fL and MRV - MSCV <25 fL are used as the threshold for diagnosing HS, the sensitivity and specificity are 84.2% and 94.7%. Arora et al.\(^36\) found that when MCV - MSCV >10 fL is used as the threshold for diagnosing HS, the sensitivity and specificity are 82.8% and 95.9%. Using an algorithm of MCV - MSCV >10 fL and MRV - MSCV <25 fL, for the differentiation of HS from immune hemolytic anemia, the sensitivity and specificity reach 68.9% and 98.8%, respectively. We select MSCV <MCV and MRV ≤95.77 fL as auxiliary indicators in the proposed new HS diagnostic protocol due to the high sensitivity and specificity, while it is convenient for clinicians to use.

2.2 | Red blood cell morphology examination

Red blood cell morphology examination is an essential test for hemolytic anemia, and peripheral blood smear indicating spherocytosis is one of the important bases for the diagnosis of HS. The morphological characteristics of spherocytes include the small size, spherical shape, deep staining, and the disappearance of central lightly stained region.\(^37\) Spherocytes in the HS diagnostic guidelines proposed by Bolton-Maggs et al.\(^9\) were only described because the cell number in the peripheral blood increased. The HS diagnostic protocol developed by Shen et al.\(^7\) describes spherocytes and/or microspherocytes as a diagnostic indicator in more detail. Microspherocytes that are deeply stained with small cell bodies and disappearance of central lightly stained region are shown in the blood smear tests. The cell number ranges from 1%-2% to 60%-70%, mostly above 10% (normal value <5%). However, there are also about 20% of HS patients who lack the typical microspherocytes. Patients with AIHA and G6PD deficiency can be accompanied by peripheral blood spherocytes.\(^7,14\)

2.3 | OFT

OFT is a semiquantitative test to detect the resistance of red blood cells in different concentrations of hypotonic saline solutions, and positive results are reported in many diseases such as HS, HE, and AIHA.\(^2,24,38\) Shim et al.\(^39\) compared HS patients with non-HS controls and showed a sensitivity and specificity of OFT in HS diagnosis of 66% and 81.8%, respectively. They also found that the use of flow cytometric fragility test (FCMOF) could improve the diagnosis efficiency, with a sensitivity of 91.3% and a specificity of 95.8%. We used FCMOF test to compare between patients with HS, THAL, and healthy controls, and regarded the percentage of the remaining red
blood cells (<23.6%) as the threshold for diagnosing HS, with a sensitivity of 85.71% and a specificity of 97.24%. Compared with OFT, FCMOF was easy to operate, time-saving, and had fewer interference factors, and high sensitivity and specificity in the diagnosis of HS.

2.4 | Acidified glycerol lysis test (AGLT)

Due to the affinity of glycerol and membrane lipids for hypotonic glycerol salt buffer with pH = 6.85, the buffer can chemically react with membrane lipids, resulting in slow hemolysis of the red blood cells, and as cell lysis increases, the level of absorbance of the solution decreases gradually. The time taken to perform the AGLT is the time taken for the level of absorbance to drop to half the initial absorbance level. A study by Bianchi et al. showed that the AGLT time of HS patients was significantly shortened, and the diagnostically sensitive 95% was better than that of OFT. However, patients with AIHA and chronic renal failure can also be positive for AGLT.

2.5 | Eosin-5'-maleimide (EMA) binding test (EMABT)

EMABT is a test in which EMA, a fluorescent dye, binds to erythrocyte membrane molecules, such as band 3. EMABT is a flow cytometric test used to detect the fluorescence intensity of EMA-labeled red blood cells. Manciu et al. reported the sensitivity and specificity of EMABT in diagnosing HS as 90–95% and 95–99%, respectively. Patients with erythrocyte membrane defects such as HE, Southeast Asian ovalocytosis, and hereditary pyropoikilocytosis may be positive for EMABT. In addition, EMA is relatively sensitive to temperature, and repeated freezing and thawing of the EMA reagent can easily reduce its fluorescence efficiency and affect the test results. Another disadvantage of EMABT is that the normal controls and HS currently established by individual laboratories lack a common reference ranges due to the different mean fluorescence intensity (MFI) scales displayed on flow cytometry from different producers. Harmonization for all clinical laboratory measurement procedures can minimize erroneous clinical, financial, regulatory, or technical decisions. However, standardization of EMABT across laboratories has been an ongoing issue. As is mentioned, a positive result for HS is indicated by a reduction in MFI reading for the EMA-labeled red cells from a patient, i.e., below the cutoff MFI value. Giving the actual MFI readings is the simplest way to report a test result, which can be a number between two and five digits due to the broad range of fluorescence scales on different models of flow cytometers. Thus, it becomes difficult to compare MFI results between laboratories. The guidelines issued by the International Council for Standardization in Hematology (ICSH) listed EMABT as one of the tests in the HS diagnostic protocol and suggests that EMABT can be combined with OFT and AGLT to screen for HS. However, the EMABT is mainly conducted in research centers or professional laboratories of medical institutions. The European Network for Rare Red Cell Anemias (ENERCA) conducted a survey of 25 research centers in Europe and found that only about 60% of research institutions can screen for erythrocyte membrane defects such as HS using EMABT.

2.6 | Gene mutation detection

HS involves a variety of gene mutations with scattered mutation sites, and there is no hot spot mutation that can be screened yet. The current analysis method used for HS-related gene mutations is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technology or real-time fluorescent quantitative polymerase chain reaction (PCR) technology. These can be used to determine the type of membrane protein defects and also to detect the mutation of corresponding genes. However, this method has disadvantages such that it is time-consuming and expensive, and is not conducive for a wide use in conventional laboratories. In recent years, the emerging high-resolution melting curve has become a genetic analysis method for detecting single-nucleotide polymorphisms. It has the advantages of rapid detection, high throughput, high sensitivity, and high specificity. When this method was used to detect the heterozygous change of a single base of a PCR product, the sensitivity and specificity were 95–100% and 99–100%, respectively. Using the high-resolution melting curve to screen for c.166A>G heterozygous mutations of HS-related SLC4A1 gene, both the sensitivity and specificity were 100%. Therefore, the use of high-resolution melting curves to screen HS patients and their families, combined with the direct sequencing methods, can quickly and accurately identify the mutation sites of disease-causing genes. However, the high-resolution melting curve has certain limitations, such as poor identification of A/T or G/C base changes, and lower detection of large fragment-amplified products than small fragment-amplified products. Next-generation sequencing is a new technology for genetic screening and diagnosis, which can measure multiple genes at a time, and has the advantages of high throughput, high speed, and high sensitivity. However, this technology cannot detect remote intron mutations, regulatory sequence mutations, and large-scale deletion mutations. Compared with conventional inspection methods, the next-generation sequencing has higher cost, and there is a need to improve the current clinical application standards, supervision, and genetic counseling.

2.7 | Simple and practical protocol for HS diagnosis

As per these existing experimental technologies and studies, combined with our research results, we propose a simple protocol for HS diagnosis.
and practical protocol for HS diagnosis in allusion to the high incidence of HS and the difficulty in HS diagnosis (Figure 1):

1. **Clinical manifestations:** typical symptoms are anemia, jaundice, splenomegaly, and a common complication is cholelithiasis.

2. **Routine laboratory tests:** Hb level is normal or decreased (trait form: normal; mild: 110–150 g/L; moderate: 80–120 g/L; severe: 60–80 g/L; Ret is normal or increased (trait form: <3%; mild: 3–6%; moderate: >6%; severe: >10%); MCHC is normal or increased; MRV is decreased (cutoff: ≤95.77 fL); MSCV < MCV; spherocytes may be increased in number; and serum total bilirubin is increased, mainly presenting with an increase in the level of unconjugated bilirubin.

3. **Family investigation:** most patients present with autosomal dominant inheritance and have the same examination results and clinical manifestations as one of their parents or other family members.

4. **Genetic testing and other screening tests:** for patients in whom the diagnosis of HS is difficult, genetic testing is required, in combination with OFT, EMABT, AGLT, Coombs test, and G6PD level determination.

### CONCLUSION

The diagnosis of HS mainly relies on laboratory tests. As per the HS diagnostic protocol, medical workers rely on MRV, MSCV, MCV, and blood cell morphology examinations when patients present with one of the following clinical manifestations: anemia, jaundice, and splenomegaly. A patient with decreased MRV, MSCV < MCV, and increased number of spherocytes can be initially diagnosed with HS. Then, the patient can be confirmed to have HS if the subsequent family investigation results (routine examination results for hemolytic anemia, including Hb level, Ret, MRV, MSCV, MCV, MCHC, peripheral blood cell morphology, serum total bilirubin and unconjugated bilirubin) and clinical manifestations reveal similar between the patient and one of his/her parents or other family members. For the patients in whom the diagnosis of HS is difficult, genetic testing and other screening tests (OFT, EMABT, AGLT, Coombs test, and G6PD determination) can be selected to confirm the diagnosis.

In summary, compared with the existing HS diagnostic protocols, the newly HS diagnostic protocol proposed by us is simple. This new protocol proposes the addition of some experimental tests (with ideal diagnostic efficiency, such as MRV, MSCV, MCV), in combination with the clinical manifestations, family investigation,
routine tests for hemolytic anemia, genetic testing, and other screening tests. Thus, this new HS diagnostic protocol could improve the clinical practice and efficiency of HS diagnosis.

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CONFLICT OF INTEREST
The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS
All authors were involved in formulation of the manuscript. Yangyang Wu and Lin Liao wrote the manuscript. Faquan Lin revised the manuscript. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
The supporting materials used in this study are contained within the article.

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