Recent developments and innovations in red blood cells diagnostics

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Abstract: Although the diagnosis of anemia is a relatively simple and straightforward endeavor, since it is mostly based on the measurement of hemoglobin in whole blood, the accurate characterization of red blood cell (RBC) disorders has emerged as a mainstay in the era of precision (laboratory) medicine. The many technological advances occurred in laboratory medicine over recent times have enabled the introduction of a vast array of innovations also for RBC diagnostics, mostly represented by modular automation, digital hematology, innovative erythrocyte parameters and adaptation of disruptive technologies to laboratory hematology, especially mass spectrometry and molecular biology. These important breakthroughs have enormously contributed to broadening the diagnostic armamentarium and making more efficient and sustainable the diagnostics of RBC disorders. Some on these important innovations will be discussed in this article.

Keywords: Hemoglobin; red blood cells (RBCs); anemia; diagnosis; laboratory

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Introduction

Anemia, which is currently defined by the World Health Organization (WHO) as a hemoglobin value in whole blood <120 g/L in women and <130 g/L in men, respectively (1), can now be regarded as a worldwide endemic disease, with an estimated prevalence of 1.62 billion people, which approximates 25% of the worldwide population (2). Albeit iron deficiency is the leading cause of anemia (i.e., approximating 50% of cases), this condition is rarely present alone, but may coexist with a kaleidoscope of other causes such as nutritional deficiencies (i.e., folate or vitamin B12 deficiencies), acute or chronic bleeding, hereditary red blood cell (RBC) disorders (i.e., hemoglobinopathies, spherocytosis), infections, renal or liver impairment, cancer and chronic inflammatory conditions (*Table 1*) (1).

Since the clinical signs and symptoms of anemia are often poorly specific, and may also be subtle, especially in patients with chronic forms of anemia, laboratory hematology represents a virtually unavoidable part of both the diagnostic reasoning and clinical decision making, since laboratory tests provide irreplaceable information for screening, diagnosis and monitoring of RBC disorders (3,4). Beside the conventional measurement of whole blood hemoglobin content, which is a necessary precondition for diagnosing anemia, the preliminary classification of anemias is usually based on the values of mean corpuscular volume (MCV) and RBC distribution width (RDW) (5), as summarized in *Table 1*. This approach is still forthright and valid, but does not enable a definitive etiological characterization, and should hence be complemented with a panel of additional laboratory investigations. Importantly, a number of technological advances occurred over the past few decades have enormously contributed to broadening the diagnostic armamentarium and making more efficient and sustainable the diagnostics of RBC disorders. Some on these important innovations will be summarized in the following

Table 1 Classification of anemia according to RDW and MCV

Condition	RDW	MCV
Nutritional deficiencies		
Iron deficiency	↑	↓
Folic acid deficiency	↑	↑
Vitamin B deficiency	↑	↑
β-thalassemia	↑	↓
Hemolytic anemias		
Immune hemolytic anemia	↑	↑
Hereditary spherocytosis	N/↑	N/↓
Anemic hemoglobinopathies (i.e., SS, SC)	↑	N
Sickle cell trait	N	↓
Chronic disorders		
Chronic diseases anemia	N	↓
Chronic liver disease	↑	N/↑
Hematologic disorders		
Aplastic anemia	N	↑
Chronic leukemias	N	N
Myelodysplastic syndrome	↑	↑
Other thalassemias	N	↓
Acute hemorrhages	N	N

↑, increased; N, normal; ↓, decreased. RDW, red blood cell distribution width; MCV, mean corpuscular volume.

parts of this article.

Automation of laboratory hematology

Laboratory automation should be regarded as one of the major advancements occurred in RBC diagnostics. Laboratory automation is conventionally defined as a multi-disciplinary integration of robotics, information technology (IT), sample handling and many other technologies aimed at developing and optimizing both workflow and activities within medical laboratories (6). Briefly, laboratory automation may bring paramount benefits to *in vitro* diagnostic testing, including easier and more efficient management of workflows, better withstanding the increasing complexity and volume of routine and urgent testing, improved turnaround time (TAT), dismissal of many manual activities, enhanced walk-away, cost savings (i.e., especially those attributable to subsidiary staff and technicians), improved standardization of procedures, lower chance of errors throughout the total testing process, decreased biological risk, along with opportunities to implement automatic reruns or reflex testing (7).

Laboratory automation has been for long limited to clinical chemistry and immunochemistry platforms. The major hurdle encountered in developing models of automation for laboratory hematology is represented by the peculiar sample type, which is whole blood anticoagulated with dipotassium ethylenediaminetetraacetic acid (EDTA) (8). The presence of EDTA in the sample, which irreversibly sequestrates ionized calcium and many other metal ions (9), makes EDTA plasma an unsuitable sample matrix for clinical chemistry and even for coagulation testing, thus generating considerable obstacles for consolidation of laboratory hematology with other branches of laboratory medicine. Nevertheless, a number of technological solutions have been developed for laboratory hematology in recent times. These basically include (I) commercialization of modular, high-throughput and versatile analyzers, which can be easily interconnected by means of sample conveyers, and can fit the organization of small, medium and large facilities, (II) integration of preanalytical workstations, which can be identical to those included in models of total laboratory automation, or can be specifically designed to suit hematological testing, (III) connection with automated slide strainers, which help improving the entire slide making process (i.e., less manual activities and lower biological risk, improved standardization of slide preparation and staining, customization of staining protocols, reduction of TAT) (10), as well as (IV) integration of automated image analysis systems (see the following section of this article). This organization is often referred to as "modular laboratory hematology" (*Figure 1*).

Digital hematology

Throughout the relatively long history of laboratory hematology, the only reliable means for identifying, enumerating and sizing blood cells has been for long represented by optical microscopy of peripheral blood smear. This practice carries many drawbacks, since it is inevitably time-consuming, is vulnerable to high inter- and intra-observer inaccuracy and imprecision, needs specific education and training of microscopists, and is poorly suited for rapid diagnostics as otherwise needed in patients with many acute hematological disorders (11).

Recent technological advances have led to development

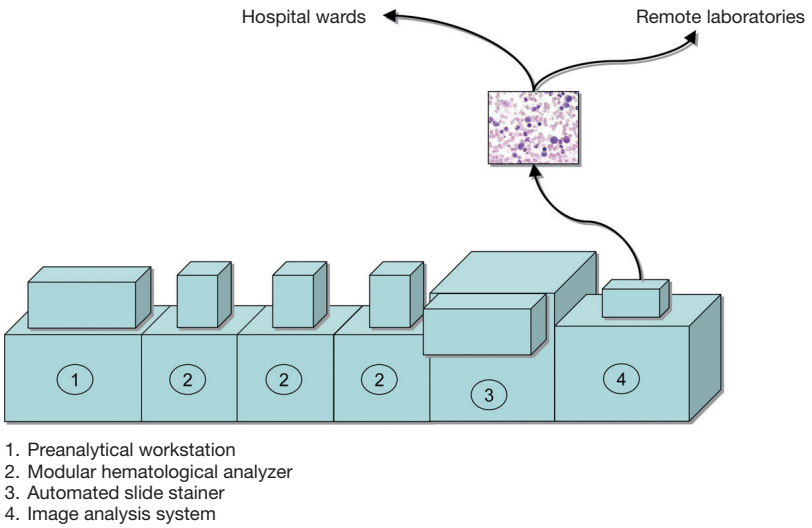

Figure 1 Modular laboratory hematology.

and commercialization of innovative automated image analysis systems, which are suited for automation and can hence be directly connected (in series) with hematologic analyzers (*Figure 1*) (12). These innovative platforms scan the slides (usually at a picture of ×100 objective), and store digitalized images of blood smears at high magnification. The images are analyzed by artificial neural networks based on a preexisting database of blood elements (thus including RBC), which can be locally customized or updated by the users. The images can be transmitted to, and displayed on, computer screens, which can be even placed at long distances from the scanner (i.e., in hospital wards or in remote laboratories) (*Figure 1*), for analysis and potential reclassification of blood elements. The operator can also increase the size of the images, or expand single sections of the scan, so obtaining a more accurate view. The operator can then accept and conserve the automatic classification, or can move elements from one cell category to another, thus improving the final reclassification. Albeit these automated image analysis systems have been originally developed for analysis of white blood cells (WBC), specific information can also be garnered on erythrocyte morphology, thus including the presence of anysocytosis, hypochromia, microcytosis or macrocytosis, spherocytosis, elliptocytosis, ovalocytosis, stomatocytosis, acanthocytosis, echinocytosis, polychromasia, poikilocytosis and abnormal erythrocytes (i.e., sickle cells and schizocytes, helmet and teardrop cells) (13). Recent data showed that the diagnostic sensitivity of these systems for identifying some critical categories of abnormal erythrocytes (i.e., spherocytes or sickle, target and tear drop cells) is excellent, typically higher than 80% (14), thus making the use of digital image analysis a highly valuable, and probably more accurate and reproducible, alternative to optical microscopy.

Notably, the use of these systems may also enable an efficient recognition of parasitoid infections such as Malaria (15), as well as the reliable identification of intravascular and spurious hemolysis, which would be otherwise undetectable on whole blood specimens (16,17). Interestingly, most of these automated image analysis systems are also capable of optimizing the identification of rare RBC abnormalities, since morphological erythrocyte alterations can be more efficiently visualized on the computer screen (18). Finally, the creation of a large personalized database of images of suggestive RBC abnormalities represents a valuable resource for education and training of students and laboratory professionals (19).

Innovative erythrocyte parameters

Irrespective of the fact that the diagnosis of anemia is relatively simple and straightforward (by measuring total hemoglobin in whole blood), the newer generation of hematologic analyzers is now equipped with many analytical and technical innovations, which enable obtaining other information than that reported with the traditional complete blood cell count (CBC), and which may ultimately provide a substantial improvement for the differential diagnosis

of anemias (20). Although a more detailed discussion will be omitted for space constraints, some important aspects deserve a special mention. These innovative parameters most typically include automated reticulocyte and nucleated RBC counts, hemoglobinization of reticulocytes and RBC, reticulocyte hemoglobin content (occasionally defined as CHr and RET-He according to the technology used for its assessment), reticulocyte maturation, automatic analysis and calculation of microcytic and hypochromic RBC (21,22). The various combination of these different parameters not only may be useful to complement clinical history, physical examination and results of more conventional laboratory investigations (i.e., CBC, ferritin, transferrin, iron, haptoglobin, folic acid and vitamin B12, among others) for troubleshooting the underlying cause(s) of anemia (23), but may also be clinically useful for diagnosing, prognosticating and monitoring other non-RBC disorders, as recently shown for patients with sepsis (24), chronic kidney disease (25) and cancer (26). The generation of complex scattergrams (27,28), which is now almost commonplace in the vast majority of hematologic analyzers, is also helpful for more accurately identifying abnormal RBC populations and other atypical elements, as recently shown for diagnosing malaria (29).

Disruptive technologies

Regardless of consolidated laboratory techniques, which have just recently made their way through phenotypic diagnostics of anemia (i.e., capillary electrophoresis) (30), a major innovation has been represented by the application of mass spectrometry and molecular biology in the diagnostics of hemoglobinopathies. The former approach allows a better characterization of hemoglobin variants preliminarily identified by screening techniques such as high-pressure liquid chromatography (HPLC) or capillary electrophoresis (31), whilst molecular diagnostic techniques enable to unravel specific molecular abnormalities characterizing many congenital RBC disorders (32).

Unlike screening tests, the selection of the most appropriate molecular diagnostic approach in patients with inherited hemoglobinopathies or RBC enzymopathies should take into account the prevalence and penetrance of the different mutations in the ethnic populations, across different geographical locations. Therefore, the first step may be represented by polymerase chain reaction (PCR)-based techniques [e.g., restriction-endonuclease PCR (RE-PCR), amplification refractory mutation system (ARMS), resolution melting analysis (HRMA), denaturing gradient gel electrophoresis (DGGE)]. Allele-specific methodologies, such as allele-specific PCR and reverse dot-blot, are especially useful for thalassemia diagnostics in target populations, enable processing a high volume of samples and are relatively inexpensive, permitting to screen some prevalent hemoglobin genes mutations at the same time. Array comparative genomic hybridization (array CGH) can then be used for detecting additional mutations which cannot be identified with first-line DNA analysis. Regarding thalassemias, gap-PCR (gap-PCR) and multiplex ligation-dependent probe amplification (MLPA) are perhaps the best options for screening and also for detecting large deletions or duplications of globin genes, which cannot be identified with conventional DNA sequencing. Sanger or next-generation sequencing (NGS) techniques may then be particularly suited for detecting all known point-mutations, but may also enable indentifying novel or rare mutations, thus helping to uncover new mechanisms of disease. A reliable guidance for the cost-effective integration of these different molecular techniques has recently been published by the European Molecular Genetics Quality Network (EMQN) (33). Notably, emerging evidence also suggests that molecular genetic testing has a pivotal role in patients with diseases characterized by clonal hematopoiesis, thus supporting the diagnostic workout of hematologic malignancies and/or myelodysplastic syndromes (34).

Conclusions

Albeit the laboratory diagnostics of anemia remains a rather simple enterprise, accurate disease characterization has emerged as a mainstay in the era of precision (laboratory) medicine, even for RBC disorders (35). The many technological advances occurred in laboratory medicine over recent times have enabled the introduction of a vast array of innovations (*Table 2*), which have led the way to a more efficient patient care and a more convenient organization of resources and workflows within the laboratory. In the foreseeable future, the better understanding of phenotypic heterogeneity of RBC disorders, also supported by IT tools such as expert diagnostic systems (36) or artificial neural networks (37), will predictably enable to improve the global management of these disorders at multiple levels. Yet, some additional issues will need to be addressed, on top of it all the current lack of harmonization in laboratory hematology (11).

Table 2 Recent developments in RBCs diagnostics

Automation
Modular, high-throughput analyzers
Integration of preanalytical workstations
Connection with automated slide strainers
Integration of automated image analysis systems
Digital image analysis
High diagnostic sensitivity
Improved identification of rare erythrocyte abnormalities
Enhanced recognition of parasitoid infections
Reliable identification of intravascular and spurious hemolysis
Possible use of the digitalized database for education and training
Innovative erythrocyte parameters
Automated reticulocyte count
Nucleated red blood cells count
Hemoglobinization of erythrocytes and reticulocytes
Reticulocyte hemoglobin content
Reticulocyte maturation
Microcytic and hypochromic erythrocytes
Low hemoglobin density
Advanced scattergrams
Disruptive technologies
Capillary electrophoresis
Mass spectrometry
Molecular diagnostics
RE-PCR
ARMS
HRMA
DGGE
Allele-specific PCR and reverse dot-blot
Array CGH
Gap-PCR
MLPA
Sanger or NGS
IT
Expert diagnostic systems
Artificial neural networks

RBC, red blood cell; RE-PCR, restriction-endonuclease polymerase chain reaction; ARMS, amplification refractory mutation system; HRMA, resolution melting analysis; DGGE, denaturing gradient gel electrophoresis; CGH, comparative genomic hybridization; MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; IT, information technology.

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