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Review Article

Analytical Phase in Laboratory - Quality Issues and Insights for Immunoassays Testing

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Abstract

The comprehensive analytical phase performance depends on many factors. Discrepant results can occur due to errors coming from outside of laboratory due to improper sample preparation resulting in the presence of hemolysis, lipids and other factors. However, standardizing testing methodology and performance characteristics such as sensitivity, specificity, accuracy, precision, and linearity is useful to ensure a final quality-based test result. Despite the benefits of fully automated processes, the quality of the analytical phase has major issues. A reported analysis of data evaluated using the Westgard rules has demonstrated that estimates on a σ scale for clinical chemistry are not satisfactory, from 3 to 4σ, at the best situation. Unfortunately, a relatively high frequency of analytical errors has been found for immunoassays with subsequent adverse clinical outcomes. Following the Good Laboratory Practice experience, we created memorable figures, called the Five Fingers (on CLIA established guidelines) and 5W rules (by ISO 15189 standardization requirements) which could be attractive to create informative and logical QA templates of each clinical laboratory procedure.

Objective: This review summarizes publications specific for immunoassay testing procedures within fully automated laboratories that follow the standardization requirement points and the best laboratory practice with intention for all staff who participates in total investigation.

Data Sources: Literature review, assays manuals, ISO standards and guidelines.

Conclusion: There are analytical performance analysis, comparison of different immunoassay methodologies, discussion of interference factors and main characteristics of calibration and quality control within clinical laboratory routine procedures focused on quality requirements discussed in this review.

Keywords: ISO Standards; Testing; Chemiluminescent Magnetic Immunoassay (CMIA)

Introduction

The analytical phase in any laboratory involves manipulations performed on samples of biomaterial to examine and obtain the final result according to the type of analysis assigned. The analytical phase starts with patient specimen journey in the laboratory until final result to be obtained and ready to report.

Advances in analytical techniques and laboratory equipment, as well as, maximizing automation process and minimizing human interactions have resulted in a decrease in error rates during the analytical phase [1].

The comprehensive analytical phase performance depends on many factors. Discrepant results can occur due to errors (more than 60% within preanalytical phase) coming from outside of laboratory due to improper sample preparation resulting in the presence of hemolysis, lipids and other factors [2]. However, standardizing testing methodology and performance characteristics such as sensitivity, specificity, accuracy, precision, and linearity is useful to ensure a final quality-based test result.

Despite the benefits of fully automated processes, the quality of the analytical phase has major issues. A reported analysis of data evaluated using the Westgard rules has demonstrated that estimates on a σ scale for clinical chemistry are not satisfactory, from 3 to 4σ, at the best situation. Other unsatisfactory analytical performance have been described also in hematology, coagulation tests and molecular biology. Unfortunately, a relatively high frequency of analytical errors has been found for immunoassays with subsequent adverse clinical outcomes [3].

This review summarizes publications specific for immunoassay testing procedures within fully automated laboratories that follow the standardization requirement points and the best laboratory practice. The article is intended for all staff who participates in total investigation process for discrepant results causing adverse outcomes, from the doctors or nurses who appoint the patient for testing, phlebotomists drawing the blood, personnel transporting the samples, and the clinical laboratory staff. Only properly trained staff involved in diagnostic process can guarantee the overall success.

Start line with the rules

Each laboratory personnel faces the same challenges focusing on the same issues. There are multiple guidelines, standardization materials and differences based on national and worldwide regulation requirements. A common view to establish methodology requirements for each clinical laboratory quality design is possible (Figure 1). Personnel must start with the basics for clinical laboratory ISO standards (ISO 15189, 2012), Clinical Laboratory Improvement Amendments [4], Clinical Laboratory Standardization Institute [5] and then evaluate according to the applicable national legislation.

Figure 1: The 5 fingers rule.

The qualified testing performance cannot be achieved without a comprehensive understanding of all laboratory processes, each with fulfilled unique design. Using the 5W rule (what, when, who, with, and why) should help personnel to create informative and logical background of each procedure (Figure 2). The offered template of the actions for testing process is described in detail in the ISO standardization requirements for clinical laboratories [6].

Figure 2: The 5W rule.

Enhancing the laboratory testing authors of this review describe the performance of immunoenzyme methodology with very common use for infectious markers and still very often appearance of the discrepancies during the analytical phase [1].

Immunoassays: from history to now

Immunoassays consist of antibody and antigen complexes to find and connect to the specific corresponding opposite substances in a patient's sample. Antibodies bind to the antigen; a specific portion of a particular pathogen [3].

Since 1973, when Rosalyn Yalow received the Nobel Prize for the idea to use special particles as the reagent components to detect antibodies or antigens in vitro, many modifications and innovations have occurred. The basic principles of the immunoassay was discovered even 20 years earlier, in 1953, when the special particles, called Ac were started to implement in laboratory reagents manufacturing process.

The earliest methods: RIA (Radioimmunoassays) and EIA (Enzyme immunoassays) were developed for practical use in laboratory [7]. RIA prototypes utilize radioactive isotopes as a label and subsequent measurement of radioactivity to measure the analyte to be detected. Two different formats: sandwich and competitive (Figure 3) to be used for antigen/antibody tests. RIA are still used in laboratories and are especially useful in measuring very low quantities of analytes. Due to its radioactive components RIA is used less often within clinical site laboratories, while another EIA types [8] are more popular and common for immunoassays to perform.

Figure 3: The earliest developed ELISA methods. Adapted from Ismail, 2009.

The advantages of Chemiluminescent Magnetic Immunoassay (CMIA) technology has been applied for routine use increasing overall sensitivity of the test. Recently being used chemiluminescent technologies with different labels such as fluorescent, radioactive and enzymatic, combining together for enzyme-multiplied immunoassay (EMIT) and cloned enzyme donor immunoassay (CEDIA) (See Table 1).

Technology	Solid phase	Separation step	Label	Detection technology
ELISA	Polystyrene, polyvinyl, nylon, glass, nitrocellulose, and silica	Wash	Alkaline Phosphatase Enzyme	Colorimetric
Description: A solid phase microparticle is coated with antibodies against the corresponding antigen or Ab or both (Ag/Ab combo assays) of interest with use to capture the analyte. The antibody for detection is labeled with an enzyme. The concentration of analyte is proportional to the amount of color measured. A noncompetitive sandwich format generates results with direct proportion to the amount of analyte present.				
Technology	**Solid phase**	**Separation step**	**Label**	**Detection technology**
CMIA	Magnetic Microparticle	Magnet	Chemiluminescent Compound	Chemiluminescent Photomultiplier Tube
Description: A chemiluminescent label conjugated to the antibody or antigen. The light is produced when combined with substrate. An advantage of this technology - the chemiluminescent reaction offers high sensitivity and ease of measurement. A noncompetitive sandwich format generates results with direct proportion to the amount of analyte present.				

Table 1: Comparison of ELISA and CMIA technologies using for detection of infectious markers.

A chemiluminescent label generates light interacting with triggering reagent. Nowadays a lot of laboratory instruments are working on chemiluminescent technology, varying only the specific type of labels.

Analytical performance for immunoassays

Clinical sensitivity and specificity terms work for the assays to accuracy and reproducibility minimizing the numbers of false results that occur for final test result [9]. Also, these two terms are typically considered as synonyms of accuracy and precision when describing the quality of the analysis. By the point of analytical view there are targeted understanding within these four characteristics of performance (Figure 4).

Figure 4: Performance characteristics. Adapted from Ward, 2017.

An assay with limited false positives is considered to be a highly specific. This can occur for many reasons, but usually assay methodology refers the limits of being measured [10]. An assay expected to perform by high clinical sensitivity term is ensuring the false negatives do not happen. From the clinical point of view the highest sensitivity without compromising of specificity is used as a golden standard of each diagnostics pattern.

Calibration process- what does it means for laboratory?

The definition of calibration means to use samples of known concentration to set analyzer or instrumentation parameters for further performance [11].

Calibrator traceability refers obtaining even a slightly different results if the same sample is tested for the same marker by two different immunoassays. Discrepancies can be a source of misinterpretation to healthcare workers monitoring their patients and causing physicians to question which result is correct and whether an error in testing has occurred. There are various reasons for not the same immunoassay results to obtain but the main cause could be the differences in specificity of each antibody.

Antibodies could bind to different epitopes with subsequent different analytical response. Usually, manufacturers do not use identical antibodies for antigens to form a complex. Calibrators should be manufactured from absolutely known reference materials, or from so called secondary reference material, assigning the corresponding values by reference method [12]. If manufacturers decide to use different reference materials methods for their calibrators production, immunoassay test result could appear very variable.

The use of metrological terms and concepts for immunoassays testing is useful to harmonize the test results in any laboratory. The Joint Committee for Traceability in Laboratory Medicine (JCTLM) is an international body that identifies reference materials and/or methods of the highest metrological requirements [13]. However, in practice some discrepancies arise, when few reference materials or methods are available to use for the same test causing erroneous test results to be generated [14]. If companies use identical calibrators, discrepant results may happen due to the differences of antibody specificity or analytical signal type. Recommendation to use identical, or basically similar, reference material and/or method for analytical performance has to be a guaranty for effective assays' harmonization, with minimal variability in results testing the same specimens by two or more methods [13].

Controls in routine analysis- impact on Immunoassays

Controls are samples that contain known concentrations of required analyte. The terms of accuracy and precision are obtained by controls use in routine practice. Controls must be included in every assay run, or day run depending on manufacturer's assays package insert requirements. Analysis is typically performed using a Levey-Jenning Chart (Figure 5) [15,19].

Figure 5: Levey-Jennings chart with variable modes of acceptance and warning examples.

Control chart graphical values indicates any potential concern or trend for the selected analyte. If any control chart is trending up or down, worth to think about the reagent, calibrator, or analyzer performance causing the issues that may affect the patient final result.

Assay interferences obtaining the final testing result

One step immunoassay can be susceptible to interferences affecting sensitivity and/or specificity. Interferences mostly occur due to the substances that interfere with the binding process of antibodies to antigens for many of reasons [9]. Prozone or hook effect occurs when the antibody being detected is in excess.

Prozone or Hook effect can falsely lower the observed value in a one-step immunoassay [9]. Too high concentrations of particular antigen in the sample bind to the available sites on the antibody-solid phase and on the antibody-labeled conjugate preventing the so called "sandwich" formation while fully saturation occurs. (Figure 6). In such cases worth to discuss human anti-mouse antibodies (see next section). The advantages of sequential assays are maximally closer to generate accurate results by their methodology of binding proteins or other interfering substances, due to the implementation of extra wash step [9].

Figure 6: Hook effect. Adapted from Gauchez, 2015.

Human Anti-Mouse Antibodies (HAMA) role

Very specific interference in immunoassays testing caused by the presence of human anti-mouse antibodies also called HAMA [16]. These antibodies can be already circulating in patient's blood as known host response after mouse antigens exposure [9]. There could be different reasons how the human's immune system produce antibodies to the mouse antigens. Patient specimens with HAMA may generate false positive or false negative results if laboratory use immunoassays with mouse monoclonal antibodies. The most popular example could be sandwich type assays (Figure 2) which usually are susceptible to HAMA interference.

In this case, obtaining false positive result, means "a sandwich is formed" and signal is generated and further detected, even though no responding analyte is present in patient's sample. The HAMA could bind either to the solid phase capture antibody or the labeled antibody in the substance of reagents (Figure 7B). Could be another scenario when HAMA causes false negative immunoassay result by binding and subsequent blocking the solid-phase capture antibody, or another way by binding and blocking the labeled antibody in the reagent (Figure 7C)

Figure 7: HAMA interferences.

Manufacturers utilize different techniques [16] to avoid or minimize the possible impact from HAMA by true two step design washing away interference or use various blockers (Figure 7A) to reduce or eliminate interference by filling the binding sites on HAMAs with other substance.

Heterophile antibodies

Another interference may be caused by heterophile antibodies which are produced against antigens from different type of origin as, for instance, animal blood [9]. The exposure occurs to animals' antigens in various ways: by inoculation with a vaccine from animal serum or by animals "in house" or surrounding. Heterophile antibodies are associated with various diseases such as mononucleosis or leukemia, cytomegalovirus infection, rheumatoid arthritis, and, of course, viral hepatitis [17].

Heterophile antibodies very similar to HAMAs antibodies interfering with immunoassays resulting mimicry final effect for

testing substance. Routinely is impossible to predict the presence of these antibodies in any sample as practically these interferences use to be unknown if patient has been exposed to any of foreign heterophilic material with subsequent immune response. Worth to mention, that any individual's immune response to any of alien material might be very different and not always the same [9].

HAMA interferences may be avoided, or minimized, as interferences by heterophile antibodies by two-step methodology design with subsequent washing effect for the antibodies before them binding or by blocking substances to use selecting the assay. Another possible way is to use sample dilutions minimizing the quantity of interfering antibodies and recalculate the final result [14]. If there is a possibility to use the system or reagents from different manufacturer or manufacturers, in any doubtful case, worth to remember all the possible interferences for immunoassays.

Discussion and Conclusion

The challenges met in routine laboratory practice are common: how to choose the right system for testing, how to compare the results, how to avoid discrepancies, and many others. Eventually, all depends on individual analytes, supplies, total need, test scope and clinical purpose. Each laboratory has a desire to get a reliable and fair result the first time evaluating the patient's sample with the assay. The rules existing in clinical practice are under accreditation, validation, and quality assessment aspects.

The best laboratory practice often use the terms "standardization" and "harmonization" defining good achievable quality results delivered to patients. The value could be achieved using harmonization understanding within structured laboratory assessment to achieve own goals [14]. A Reference laboratory definition is a synonym of the best quality with an indisputable and genuine final result. An important aspect of harmonization is subject to change. Commutability is defined as a property of a reference substance for which the closeness of the ratio of the results obtained for a particular substance is finished by applying two different measurement procedures obtained from the measurement results of other specified substances [18].

The aim of the Joint Committee for Traceability in Laboratory Medicine (JCTLM) is to compare the results of research with different methods ensuring optimal medical decision optimal health care. For that purpose, the Reference Measurement System (RMS) which includes the initial analyte definition, the Reference methodology for specific measurement, primary and secondary reference materials and Reference Institutions – Laboratories, should be considered for use in the laboratory [14].

The use of practical research materials or known published achieved good practice results are always useful for disputable results to obtain own way to resolve. Scientific and clinical basis of each investigation or assay is always a priority. Each laboratory quality decisions and exact procedures for all the processes is critical to achieve a reference reportable value. Analytical assay performance to its desirable approach means the correct actions manner and design from the beginning first.

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