INTERFERENCE IN IMMUNOASSAYS

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ABSTRACT

Immunoassays are a simple, efficient and reliable means for testing patient samples in the modern hospital. Typically, compared to their alternatives, they are cheap, specific and sensitive tools that have provided robust means by which the detection of analytes (antigens) can be achieved easily. The underlying principle in immunoassays, that of antigen-antibody reactions forming a measurable complex is what exposes immunoassays to possible interference from proteins and other reactants with structural similarities to the target epitopes in the analytes. Despite their remarkable utility and widespread application in hospital laboratories, the impact of interferents both endogenous like autoantibodies and exogenous factors like drugs, on immunoassays is worth investigating since the alternative would be producing unreliable results. Given that 70% of all diagnostic decisions rely on laboratory results the consequences of interference in immunoassays can be catastrophic to the healthcare sector. Insights for developing viable tools for assessing discordant patient results; including troubleshooting steps like repeat testing with alternative methods and creating escalation procedures between clinicians and laboratorians for case-by-case reviews of suspicious results have been highlighted. In addition to the incorporation of statistical tools, the potential use of Artificial Intelligence as a possible remedial measure has been proposed too.

Keywords: Endogenous, Exogenous, Immuno-assays, Immune complex, Interference.

İMMUNOANALİZLERDE ENTEFERANS

ÖZ

İmmünoanalizler, hasta numunelerini test etmeye yarayan basit, verimli ve güvenilir araçlardır. Tipik olarak, bu analizler, alternatifleri ile karşılaştırıldığında, analitlerin (antijenlerin) tespit edilmesini kolaylaştıran hassas, ucuz ve spesifik testler olarak öne çıkmaktadır. İmmünoanalizlerin altında yatan prensip, ölçülebilir bir kompleks oluşturan antijen-antikor reaksiyonları olup aynı zamanda immünoanalizleri analitlerdeki hedef epitoplara yapısal benzerlikleri olan proteinler ve diğer reaktanlardan olası müdahaleye maruz bırakan şeydir. Hastanelerde sıkça kullanılan immünolojik testlerin birçok faydası olmasına rağmen, endojen otoantikorlar ve ilaçlar gibi bazı eksojenik faktörlerin bu testler üzerindeki etkisi, güvenilir olmayan sonuçlar alınmasına neden olabilmektedir. Diyagnostik kararların %70'inin laboratuvar analiz sonuçlarına göre alındığı düşünüldüğünde, immünolojik testlere müdahale edilmesinin sağlık sektörü için yıkıcı etkiler yaratabileceği unutulmamalıdır. Bu sebeple uyumsuz hasta sonuçlarını değerlendirmek için uygulanabilir yöntemler geliştirmeye yönelik çalışmalar yapılmaktadır. Bunlar alternatif yöntemlerle test tekrarının yapılmasını, şüpheli sonuçların vaka bazında incelenmesini, klinisyenler ve laboratuvarlar arasında ileri seviye prosedürlerin oluşturularak sorun giderme basamaklarının uygulanmasını içerir. Ayrıca istatistiksel yöntemlere ek olarak, yapay zekanın potansiyel olarak kullanılması da olası iyileştirici önlemler olarak önerilmektedir.

Anahtar sözcükler: Endojen, Eksojen, İmmüno-analiz, immün kompleks, Enteferans

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INTRODUCTION

Immunoassay technology was developed and refined over time to its state currently. The adaptation of these assays into the clinical setup for quantitative assessment of analytes during this time spearheaded the emergence of dozens of various types of immunoassays. In a nutshell, they comprise techniques like turbidimetric and nephelometric assays, surface plasmon resonance–based immunoassays, and labelled immunochemical assays among others(1–3).

The jump to labelled immunoassays was chaperoned by Yallow and Berenson's radioimmunoassay. The pair were studying retarded insulin secretion in diabetic patients. These patients at the time were being injected with bovine insulin, as a result, the immune response against this insulin resulted in antibodies binding the insulin and preventing its secretion from urine(4). To compare the insulin levels across their intended study group they had to devise a test capable of detecting low levels of insulin. Thus they labelled the target antibodies with radio-isotopes and quantified their results by detecting the subsequent radiation upon the radio-labelled antibodies binding to insulin in patient samples and creating antigen-antibody complexes that were quantifiable (3).

Milstein and Kohler's breakthrough in developing monoclonal antibody technology among other advancements eventually provided alternatives that ultimately birthed the replacement of radioisotope labels with enzymes and chemiluminescence. All this progress made immunoassays cheaper, more efficient and safer thus significantly contributing to their pervasive adaptation for diagnostic purposes (2,3).

Immunoassays since then have seen tremendous development mostly by benefiting from advancement in immunological knowledge accompanied by synergistic leaps in other fields (5). These developments paved the way for the automation of immunoassay processes and vastly improved the sensitivity of these assays thus allowing for their expanded use in diagnostics with the added benefit of incorporation of faster turnaround times, and automation of patient management (1). The continued widespread use of immunoassays for both research and diagnostic purposes shows the importance of immunoassays and the need to investigate how they are impacted by interference (6).

Considering the multidisciplinary nature of the advancements in immunoassays, we sought to identify and discuss the causes of and solutions to interference in-clinical diagnostic labs bearing in mind the role of instrumentation and automation in this context. Given that immunoassays play a critical role in the analysis of many laboratory analytes of clinical significance, the causes of interference in immunoassays must be assessed since possible solutions for these problems might be useful(7).

TYPES OF IMMUNOASSAYS

The radio-immunoassay derived its name from the use of radioisotope Iodine as a label tagged to an antibody which then binds to its target antigen (3). On the other

hand, chemiluminescent and fluorescent immunoassays are labelled with luminous signal-emitting chemicals for the former and fluorophores for the latter which get triggered in the event of an immune complex formation. In addition to the mentioned techniques, enzyme-linked labels also do exist as well as new and emerging labelling techniques exploiting the antigen-antibody reaction principles(1).

As such the classification of immunoassays was developed broadly based on how assays are labelled and the signal detection method employed. Immunoassays do make use of chemically attached or conjugated antigen or antibody labels like radio-isotopes, enzymes, chemiluminescent molecules or label-free methods. The latter group an example of which is Surface Plasmon Resonance (SPR) based Immunoassay, a technique which is very useful in the characterization of binding reactions in real-time, measuring endpoints such as antibody affinity, kinetics and cross-reactivity(8).

This method principally relies on SPR, an optical phenomenon which measures the refractive index changes around thin metal layers preferably gold following a biomolecular interaction like in the case of an antigen binding to its antibody. The mechanics of this technique involve immobilizing the antibody onto the gold surface, over which the reactants flow. Light is then made to interact with the gold surface resulting in the surface producing electron charge density waves called plasmons at the sample and gold surface interface causing a reduction in the intensity of the reflected light (8,9). These slight changes in the refractive index at the interface cause a signal change, thus facilitating real-time detection of surface molecular interactions via this technique as reactants bind and unbind to each other. These characteristics have made this technique an essential diagnostic tool for the monitoring of monoclonal antibody drugs(10).

Aside from SPR, other label-free immunoassays do exist and they make use of different detection methods that also do not require labelling or modification of assay components (11). The idea behind choosing the desired label for measuring a particular analyte is primarily based on the safety and sensitivity of the label, hence label-free immunoassays in this case tend to offer a wider dynamic range, increased sensitivity and specificity of the assay(5,10).

Despite the ubiquity of labelling tools in addition to alternatives like label-free assays. Immunoassays are still subject to interference in principle based on the mechanism they employ for the quantification of analytes which involves the formation of an antigen-antibody complex between the desired analyte and their target antigen and/or antibody(1).

An added layer of complexity emanates from the automation of most immunoassays thus introducing interference derived from the methods employed in the detection of the measurable immune component (12).

INTERFERENCE

Immunoassays are at the core of the modern diagnostic laboratory because of the versatility of antibodies with regard to the enormous potential to bind with pinpoint specificity to their target molecules. That

combined with increased detection ability from employing the specificity of modern labels makes immunoassays indispensable for the modern laboratory and medicine (13)

By definition interference is the presence of a substance in the sample that alters the true value of the result., the key causes of interference are discussed below. Nonetheless, the causes of interference in immunoassays are as numerous as they are diverse (14,15). To fit these key concepts within the scope of this paper they will be classified as endogenous that is those factors that are analyte associated and exogenous that is those factors that are analyte independent as per the below Table 1;

Table 1: Classification of factors associated with immunoassay interference

Analyte-associated interference/endogenous factors	Analyte-independent interference/exogenous factors
Key factors. -Cross-reactivity of reactants -Endogenous antibodies Secondary factors. -Hook effect -Binding proteins -Idiopathic -Preanalytical (Drugs, etc.)	Pre-analytical errors during sample preparation. -Improper Centrifugation -Hemolysis, lipemia, icterus -Carryovers. Analytical errors occurring during analysis - Inadequate separation from binding proteins. -Antibodies directed against. -Interference with signal generation via therapeutic ingestion of agents like biotin.

The key reaction that defines an immunoassay is the antigen-antibody reaction, the principle being the specificity of the antibody for its antigen. However, considering that similarities do exist between various proteins then we can infer that there is a possibility of factors present in the analyte with similar molecular structures cross-reacting with the antibody instead of the targeted analyte. Which is a major cause of concern for hormonal assays (16,17).

In these assays, the source of cross-reactivity may be associated with the antibody used or from the targeted antigens cross-reacting with other analytes in the sample. Studies have reported significantly persistent cross-reactivity in the cortisol assay with prednisone although at varying levels (18). In this case manufacturers of these kits attempt to mitigate the effects of the cross-reactivity by tweaking the design of their kits to reduce the levels of cross-reactivity although most specialist centres opt to go for alternative testing methods like High-performance liquid chromatography (HPLC) where the analyte in question may be susceptible to cross-reactivity(15).

Endogenous antibodies

These are innate antibodies with specificities against one of the reactants in the assays. Studies have identified these types of interferents and have classified them as heterophile antibodies, Human anti-animal antibodies (HAAA) and auto-antibodies (17,19).By order of significance in terms of their ability to cause interference heterophile antibodies are ranked lower in comparison to the latter two classes of antibodies. The reason is that these naturally occurring non-specific

heterophilic antibodies are easily replaceable upon antigen exposure because they bind to the antibody weakly with low affinity usually interacting with the Fc region of immunoassay antibodies. Heterophilic antibodies are a rung lower as endogenous interferents when compared to HAAA's which are specific and interact strongly with assay antibodies (20–22).

Humans do develop HAAA's because of exposure to antigens from animals, from treatment with therapeutic antibodies or close association with animals in the environment. Murine antibodies are most common and they are used most often in assay reagents hence most people would likely have anti-mouse antibodies considering the ubiquity of rodents in human habitats (23–26).

Autoantibodies by definition develop from autoimmune reactions and the antibodies result from the body producing antibodies with specificities against self-antigens. This is commonly seen in rheumatoid arthritis and patients with thyroid disorders developing anti-thyroid antibodies. Studies estimate that a quarter of all adults express Rheumatoid Factor (RF) which is an interferent that affects several immunoassays (27,28).

Secondary factors.

The hook effect results in false negatives or inaccurately low results because of the inability of antibodies to bind antigens and form immune complexes which can then be detectable. It occurs in immunoassays with either antigen excess or antibody excess. It is common in cases where analytes may be present in the sample at particularly high concentrations for example in human chorionic gonadotrophin hormone (HCG) testing (29).

Interference caused by secondary factors like the hook effect or binding of a non-analyte substance and other idiopathic causes may be harder to factor into the design of the immunoassays, as such assays rely on the person(s) doing the test to troubleshoot their assays. Doing this can be accomplished via trial and error. Establishing that an error occurred in the first place can be done by looking at the results to pinpoint the potential presence of interference and the possible nature of the interferents(30,31).

The best indicator for interference is discordant results, and from the results, it is possible to troubleshoot the problem with the assays. For example, looking into a discordant result for the beta-HCG test used to detect pregnancy in females and malignancies in males in the physiological state of pregnancy and disease states for these conditions respectively. The detection of the beta-HCG outside of the expected clinical settings should therefore be examined carefully considering the life-changing nature of this test in the circumstances described (32,33).

Analyte-independent interference/exogenous factors

These contribute to pre-analytical errors and the causes of interference at this point are mainly the consequence of human error, hence with standardised proper operating procedures and automation, they can be mitigated and reduced significantly(32).

The most prevalent preanalytical errors include; sample identification errors, clotted, haemolysed samples, inaccurate

analysis request forms, and faulty transportation and storage. This is in addition to improper sample collection including the use of incorrect sample tubes. The impact of these avoidable sources of interference can be massive in terms of cost and no effort should be spared toward their eradication. In case of suspicion of interference, they should be isolated and ruled out before conducting any further investigation for any other sources of interference (34)

Human error-linked interference as it relates to immunoassays occurs disproportionately during the pre-analytical phase and where automation is deficient, during the analytical phase. The potential for carry-over of proteins either in the sample tubes or from improper cleaning of analytical instruments also creates possible sources of interferents during the analytical phase. As seen especially when the results for an analyte are extremely elevated, the washing steps should be queried. While modern instrumentation has inbuilt mechanisms to account for sample hemolysis, lipemic samples, icteric samples and carry over's it is still imperative to rule out these analyte-independent factors in cases where results are inconsistent with the clinical picture(12,31,35).

Finally, the use of biotin for therapeutic purposes has contributed to false results in sandwich and competitive immunoassays and should be considered as a possible interferent in immunoassays using biotin-streptavidin in case of discordant results (36,37). Attempts to circumvent biotin interference by the use of manufacturer-produced biotin-suppressed immunoassays do not adequately eliminate the effects of this interference. Additionally, since there is no routine determination and or exclusion of biotin from patient samples before an immunoassay, makes it is difficult to weigh the effect of this interference on the test result (36,38,39).

Possible Solutions to interference in Immunoassays

Considering that most commonly seen interference occurs in the preanalytical phase attributed largely to human error; ranging from misidentification of samples, use of invalid tubes, and invitro hemolysis among others. Therefore eliminating these errors via proper implementation of good laboratory practices and automation of routine error-prone bottlenecks where possible will greatly reduce the frequency of their occurrence(35).

Interferants that impact the analytical phase on the other hand are dangerous and difficult to identify via a systematic approach and hence often require a case-by-case review of patients' results to isolate these sources of interference(15) Despite the difficulty in detection of analytical phase interferents they contribute significantly to the frequency of interference occurrence in immunoassays that reportedly ranges between 0.4 to 4%(13)

The obtaining situation in most clinical and research laboratories as it relates to immunoassays has been the mitigation of possible interferents by assessing for their effects on the results produced after analyzing the analyte. This is aided by established guidelines that come as a clear indication on the kits as to what the test can be used for and within which ranges are the results useful. In research laboratories, it is usually the researchers that set the

parameters for their in-house kits. Nonetheless, developments on ways and means to reduce interference have increased and so has the number of interferents that keep being discovered as the immunoassays themselves continue being iterated with by various stakeholders(31,40).

Among the most promising means for combating interference, is the production of heterophile blocking tubes, making use of serial dilutions to obtain the desired levels of analyte and avoid the hook effect. The standardization or even automation of pre-analytical steps like sample collection through advocating for the use of appropriate tubes for appropriate samples is also useful. Detecting discordant results in clinical labs via the implementation of a two-step verification of results before their release would also be essential in arresting any inconsistencies early(15,28).

Additionally, of critical importance is the use of statistical tools and employing recent advances in artificial intelligence that have given birth to incredible machine learning tools that could be very useful in the identification of analytical stage interferents. Machine learning could be incorporated into various analytical instruments at the result evaluation steps harnessing the unlimited computing power available currently to train algorithms capable of discerning systematic interference in real time during analysis (13,40,41).

Establishing lines of communication with the clinical staff would help in giving the laboratory an accurate picture of the patient's status to aid in matching the results obtained. Overall whenever the results are suspect, using different testing protocols where possible or more sensitive methods should be considered as a means to test the accuracy of suspicious results obtained by immunoassays(13,41).

CONCLUSIONS

Immunoassays being widely used for various diagnostic purposes and prognostic monitoring in a wide array of tests including but not limited to analysis of hormones, tumor markers, drugs, cardiac troponin, therapeutic monoclonal drug therapy and microbial serology means that the potential effects of discussed interference are considered iniquitous, dangerous to patient care and costly. From inception, these assays have been useful for the match towards the current high throughput assays in clinical laboratories. Saving costs, and time and increasing the accuracy of diagnostic results. The efficiency gained from the specificity of these tests has also made the assays a very important toolkit in disease diagnosis.

Interference, on the other hand, brings forth a credible threat as to the reliability of the results obtained via immunoassays for diagnostic and/or prognostic purposes in cases where troubleshooting the causes of discordant results fails. However, identifying inconsistencies in the results still rests on the individual conducting the test where applicable using existing mechanisms for troubleshooting the immunoassay protocols to identify the cause(s) in most cases. Thus, the reason immunoassays still retain their usefulness in clinical and diagnostic laboratories is due to the level of progress made in the means of mitigating interference through the use of statistical tools for inference and evaluation of atypical results.

It would be of greater value to have cost-effective alternative tests that investigate the possibility of interference. Additionally, due to the match towards greater automation in laboratories and the advent of big data sets, incorporating artificial intelligence in the analysis of laboratory results could improve the systematic detection of interference. We suggest that future work on these lines of inquiry could be useful.

Declaration of conflict of interest

The authors have no conflict of interest to declare.

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