Erroneous laboratory results: what clinicians need to know

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ABSTRACT – Laboratory tests such as 'conventional biochemistry' are analytically robust and trusted, however, some common tests performed by immunoassays, eg thyroid function tests, are inherently more prone to analytical interference, giving rise to incorrect results. Interfering antibodies capable of causing potentially misleading results in immunoassay varied from about 0.4% to 4%. Furthermore, this form of interference cannot be predicted *a priori* and cannot be detected even by most stringent laboratory quality control assurance schemes because it is unique to an individual sample. Since more than 10 million immunoassay tests are carried out yearly in the UK alone, the impact of this problem on delivering appropriate patient care can no longer be ignored. Clinicians tend to perceive all laboratory data in the same light. Because of this, increased awareness of the inherent limitations of these laboratory tests should trigger a more measured and thoughtful approach, thus ensuring patients receive appropriate investigations and treatment.

KEY WORDS: erroneous analytical results, immunoassay, interference, laboratory tests, wrong laboratory results

Introduction

The clinical laboratory uses many technologies for providing a wide array of tests. For example, in biochemistry some technologies utilise highly specific straightforward chemical and physical constants unique to the moieties under measurement, eg ion-selective electrodes for electrolytes or use highly specific substrate-enzymatic reactions, eg glucose, urea, and cholesterol. These technologies are generally robust, yielding analytically reliable results for most routine conventional tests such as electrolytes, liver function tests, lipids and glucose. False results in many of these tests are rarely analytical and in almost all cases are due to pre-analytical/administrative errors.[1]

The same, however, cannot be said for some tests performed by immunologically based technology such as immunoassays,[2–6] not only in biochemistry, the main provider of immunoassay data, but also in other pathology departments such as immunology, haematology and microbiology. Tests performed by immunoassays are shown in Box 1. Although results obtained by immunoassays are inherently more prone to interference and have led to misdiagnosis, treatment for phantom diseases and unnecessary surgery,[3,4] they are generally perceived by clinicians in the same light as other routine conventional tests.

The purpose of this review is to show why immunoassays are prone to interference and consequent inaccurate results. To help illustrate the spectrum of these problems and how such results can be misinterpreted in clinical practice, the following four common tests, each highlighting specific features, will be discussed in some detail:

- thyroid stimulating hormone (TSH)
- rheumatoid factor (RF)
- carcinoembryonic antigen (CEA)
- cardiac troponins (cTn).

Finally the various telltale clinical and biochemical signs of interference that may help clinicians and laboratory analysts develop a more effective strategy against such potential insidious sources of error will be described.

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Box 1. Common tests performed by immunoassays. Although this list does not include all immunoassay tests, it highlights the wide range of analyses predominantly performed by this technology.

- All endocrine tests (eg pituitary, thyroid, adrenal, parathyroid, pancreatic and gonadal) and human chorionic gonadotropin
- Tumour markers, eg carcinoembryonic antigen, prostatic specific antigen
- Cardiac biomarkers such as troponins and myoglobin
- Rheumatoid factor
- Allergy and allergen-specific IgE
- Vitamin B12/folic acid
- Specific serum proteins such as ferritin, α-fetoprotein, sex hormone binding globulin
- Specific antibodies against bacteria/viruses, eg hepatitis, HIV, cytomegalovirus, rubella, syphilis
- Anti-endocrine gland antibodies, eg thyroid peroxidase, thyroglobulin, intrinsic factor, adrenal
- Therapeutic drug monitoring such as digoxin, gentamicin, cyclosporine
- Drugs of abuse, eg cannabis and opiates

Why are immunoassays inherently prone to interference?

The immunoassay is fundamentally an *in vitro* immunological binding reaction between the molecules to be measured (ie antigen) and a reagent antibody.[5,7] This test reaction is similar to numerous highly-specific immunological interactions that take place *in vivo* in healthy individuals. Interference in immunoassay is essentially caused by an inappropriate and undesirable 'cross binding reaction' which may be analogous to those which occur *in vivo* causing some autoimmune diseases. In immunoassays, such undesirable *in vitro* 'cross reactions' causes analytical error while *in vivo* it causes autoimmune pathology.

The binding reaction between an antigen and an antibody utilises a number of long- and short-range forces of attraction which are fundamentally dependent on the three-dimensional shape of the molecule plus the electron cloud at the binding site(s).[5] It is therefore important to recognise the difference between laboratory analyses using immunological binding mechanisms that primarily recognise shape and other technologies that use physical constants and/or use highly specific chemical or enzymatic reactions unique to each molecule.

Molecules with similar shapes may therefore bind to the antibody. In fact, the concept of monospecificity in immunoassay is no longer tenable.[5] Lack of monospecificity is, in certain cases, recognisable and predictable and as a result, laboratory analysts are aware of them because of the well known molecular similarities, eg insulin and pro-insulin, prolactin and macroprolactin, testosterone and dihydrotestosterone, and digoxin and digitoxin.

Some endogenous antibodies, however, may be polyreactive especially in early immune responses.[5] Polyreactive antibodies tend to have lower specificity, bind to many antigens, potentially causing interference in their measurements. Even more specific antibodies may also exhibit molecular structures on their surface which may by sheer chance mimic an immunological binding site of the measured compound (ie similar epitope), a binding site on the reagent antibody (ie similar paratope), both (ie in idiotypic immune response) or to the bound antigen–antibody complex (ie metatope).[5,7] In all such cases, interference may occur leading to erroneous results (falsely high or low). Screening every sample prior to analysis for interfering antibodies is, however, neither specific nor practical.

The binding affinity/avidity (ie the strength of binding between antigen and antibody) varies from one antibody to another. It is measurable and expressed mathematically as 10^* (ie 10 to the power of the digit). The variations between these affinities in immunoassays could be several orders of magnitude. The difference between the weakest at 10^7 and the strongest at 10^{10} is actually 1,000-fold.

If, for example, the endogenous interfering antibody has an affinity of 10^7 while the immunoassay reagent antibodies have an affinity of 10^9, ie 100-times stronger, then the ability of endogenous antibody to interfere is reduced. However, if the endogenous interfering antibody has a comparable or even a higher affinity than the reagent antibody it would in all probability produce a meaningless and erroneous result. The presence of interfering antibodies may therefore cause analytical inaccuracy of different magnitudes in different methods.

What happens analytically if a serum sample contains endogenous interfering antibodies? The magnitude of interference will be dependent on the affinity/avidity of endogenous interfering antibodies as described above as well as their concentrations in serum (ie titres), both of which may vary with time. Interference may be transient over weeks (the half-life of immunoglobulins is about one month) arising from antibodies produced as a result of bacterial/viral infection, recent immunisation, treatment with monoclonal antibodies, a blood transfusion, or may be chronic in patients exposed to animal proteins (through pets or via occupational exposure) or those with autoimmune disorders.[2,5] It is generally easy to suspect interference when it is gross and without obvious clinical correlates. Interference may also affect more than one immunoassay, in highly unpredictable ways.[4] When it is modest, subtle and/or plausible, however, it may be highly misleading.[3,4,5] Box 2 highlights conditions when interference should be suspected.

Incidence of interference

The cited incidence of interference in immunoassay varied from about 0.4% to 4%[4,6] although a figure of 0.05% has been suggested based largely on strong circumstantial evidence. These differences may be attributed to the specific nature of interference, its means of identification and the study design. In the largest prospective study involving more than 5,000 patients in whom thyroid function tests were requested, however, the incidence of interference in thyroid stimulating hormone (TSH) immunoassays with potentially adverse clinical effect was found to be 0.4% of these patients.[4] The overall incidence of interference was, however, slightly higher at 0.5%.

Interference in immunoassays analyses and its clinical implications

The following selected examples highlight the variety and spectrum of interference and their various clinical sequelae.

Thyroid stimulating hormone

Thryoid stimulating hormone is probably the most commonly requested immunoassay test. This hormone is usually requested with other complimentary axis parameters such as thyroxine (T4) and/or triiodothyronine (T3) (total or free).

In subclinical hypothyroidism, normal levels of circulating T4 and/or T3 are associated with an elevated TSH. The magnitude of TSH elevation that justifies treatment, however, has been a subject of relentless interpretation and debate.[8,9] In two recent and large scientific consensus reviews on this topic involving eminent thyroid specialists from the USA and the UK, a TSH cut-off level of 10 IU/l has been suggested, below which treatment may not be justified.[8,9] Positive and negative interference from endogenous antibodies, however, causing falsely elevated

Box 2. Conditions in which the incidence of interference may be higher and should be suspected.

- Clinically unexpected
- Inconsistent with other clinical correlates
- Inconsistent with other biochemical parameters such as in multiple parameters testing, eg thyroid or gonadal axis tests in serum or serum versus urine results
- Odd results in more than one immunoassay
- Significant and unexplained change from previous test
- Patients with autoimmune or chronic diseases
- Recent immunisation, blood transfusion or receiving monoclonal therapy
- Among veterinarians and those who come into contact with animals
- Repeat analysis by a different method shows reverse or exaggerated analytical bias
- Disproportionate results on dilution and repeat testing
- Discrepant results after the use of 'blocking antibodies'

or reduced TSH could risk unnecessary and potentially life-long treatment in some patients while denying treatment for others with genuine hypothyroidism. Two published cases[4] will be discussed to illustrate the impact of positive interference using a predetermined analytical TSH cut-off level.

In the first case,[4] TSH was raised at 22 IU/l (normal range 0.2–4.0) with normal free T4 (fT4) and free T3 (fT3) suggestive of subclinical hypothyroidism. Repeat analysis using another method confirmed the raised TSH (11.0 IU/l) giving a false assurance to the clinical endocrinologist. Endogenous interfering antibodies, however, were subsequently detected. By then the patient had been subjected to 18 months of unnecessary investigations (including computed tomography scan for suspected pituitary thyrotrophinoma) and treatment for a phantom hypothyroidism. The luteinising hormone level was found to be consistently raised (>80 IU/l) on three separate occasions while follicle stimulating hormone levels were found to be within the normal range, consistent with the patient's age and normal menstrual history. Analytical interference from endogenous antibodies in TSH and LH was subsequently confirmed but FSH was not affected.

In the second case,[4] the patient had a normal fT4 and fT3 but a TSH >100 IU/L. Because of such disproportionate TSH elevation, a second method was used which showed a much lower TSH result of 8.2 IU/l. Surprisingly, however, progressive dilutions of serum samples followed by repeat analysis using the second method revealed a progressive increase in TSH levels in the diluted samples instead of the anticipated lower readings, a telltale sign of interference. These two cases show that interference is method-dependent and can produce TSH results of vastly different magnitude, potentially making some TSH-based decisions erroneous.

Negative interference in TSH has also been demonstrated and has masked potentially significant hypothyroidism in a pregnant woman and in an elderly patient.[4] In these cases, low levels of circulating T4 and T3 were associated with falsely reduced TSH levels, masquerading as non-thyroidal illness or pituitary hypothyroidism.

Rheumatoid factor

Rheumatoid factor (RF) has a central role as an aid in the diagnosis of rheumatoid arthritis (RA), and the assay for RF remains among the most commonly requested tests in rheumatology.[10] Rheumatoid factors are antibodies directed against antigenic determinants on the Fc fragment of IgG. RF has been reported to occur in 70–80% of patients with confirmed RA. Of all the known RA biomarkers, RF is the best predictor of aggressive disease.

Methods for the measurement of RF detect primarily IgM rheumatoid factor and most immunoassays for RF are optimised to quantitate this immunoglobulin class. However, IgA, IgG and IgE rheumatoid factors (as well as subclasses eg IgG-1, IgG-2) have all been described in patients with RA and have been linked with certain features of RA. There is also evidence that within an individual patient isotype switching can occur, increasing further the heterogeneity of RF in serum.

A negative or low result for IgM RF, though analytically correct, could confuse diagnosis if the patient has a different class of RF antibody. A falsely elevated antibody result caused by positive interference from another unrelated antibody has also been reported.[11] It is therefore possible that a positive RF caused by interference could confuse the diagnosis of RA.

Rheumatoid factor is commonly regarded as a heterophilic antibody (ie an antibody with promiscuous binding tendency towards other different molecules/analytes) capable of interfering in the measurement of many other immunoassays. It has long been recognised that RF is sometimes produced after viral and bacterial infection in healthy individuals. These RFs are usually polyreactive IgM antibodies of low titre (<1:80 titre). They can be detected in 1.3–4.0% of the Caucasian general population and 15–20% of elderly subjects with chronic disease.[12] Immunoassays, irrespective of its nature are susceptible to error from such circulating RFs.[13,14] More care is therefore warranted in the interpretation of all immunoassay results in patients with circulating RF.

RF is therefore a good example to highlight the effect of heterogeneity of an analyte on immunoassay results with the potential to confuse diagnosis. It also highlights the ability of RF itself, as an antibody to interfere in other immunoassays performed on RF positive patients to also produce wrong results which again could confuse diagnosis/treatment.

Carcinoembryonic antigen

The main utility of tumour markers is prognostic, monitoring disease progress over long periods of time.[15] A carcinoembryonic antigen (CEA) concentration above a predetermined cut-off limit would be suggestive of relapse while concentrations below such threshold would be consistent with remission. Interference from endogenous antibodies could cause a falsely elevated result of a tumour marker, triggering alarm and unnecessary investigation/treatment or an erroneously low result, falsely implying remission despite disease activity.

Carcinoembryonic antigen is a verified and useful tumour marker in the surveillance of patients with diagnosed colorectal cancer.[16] The incidence of immunoassay interference in CEA data leading to both false positive and negative results reported by Bjerner *et al* of the central laboratory in Oslo was 4%.[6]

An important issue which could further confuse the interpretation of many tumour marker results, including CEA, is a change in the immunoassay method. This may occur when clinical laboratories renew and update their equipment. Changing analytical equipment is invariably associated with changes in immunoassay formats, reagents and methodologies. Although such changes can be revalidated and streamlined for molecules with well-established structures and well-defined identities/ purities, eg hormones and drugs, this process is more difficult for moieties such as CEA, a high molecular weight mucin with complex array of epitopes.

The assumption is that interpretation of results could still be made in the context of the new method reference range. Such assumption may, however, be an over-simplification since a method 'reference range' is a statistical range based on samples taken from 'normal' individuals free from disease. The ability of an immunoassay to detect a relapse of a tumour producing CEA would be dependent on the nature of the CEA epitope(s) expressed by this particular tumour and the ability of the reagent antibody to interact and accurately quantitate its concentration.[17] Because tumour CEAs are immunologically heterogeneous, the reactivities of various forms in immunoassays may not be equimolar and a change in immunoassay methodology could therefore yield analytically and numerically different results.[17] Care in the interpretation of any tumour markers with multiple epitopes may therefore be paramount when the method of analysis is changed.

Cardiac troponin as an immunoassay for acute coronary syndromes

Cardiac troponin (cTn) is widely accepted as the gold standard diagnostic test in the management of patients with acute coronary syndromes without ST segment elevation and myocardial infarction.[18] It is increasingly used also in risk stratification and in non-coronary diseases to indicate prognostically important cardiac damage. The need for a quick turnaround time (about 60 minutes) for cTn results necessitates the use of immunoassay technology.

A serum troponin concentration exceeding the 99th percentile of a reference control group in a patient with chest pain is considered consistent with the diagnoses of myocardial infarction/necrosis irrespective of any electrocardiographic changes, or excludes it if it is within the 99th percentile. A falsely elevated concentration may result in a patient's hospitalisation and subject the patient to unnecessary investigations such as coronary angiography, while a falsely low result in a patient with an acute coronary syndrome may deny the patient the necessary investigations and treatment with possible adverse clinical sequelae. False positive case reports have been widely reported in recent literature though understandably, the number of false negative case reports is lower.[19,20] It is important also to emphasise that the effects of interference, either negatively or positively, may persist for weeks (or longer) because the half-life of immunoglobulins is relatively long (about one month). Protocols such as repeat analysis after 6–12 hours or 6–18 hours from onset may be unhelpful or even misleading if the initial reading is low because interference causing falsely low troponin would persist, reinforcing the negativity and giving a false sense of reassurance despite the presence of cardiac pathology.

How analytically accurate and robust are cTn immunoassays performed by the laboratory or at ward level as a 'point of care' test? Circulating cTn is not a single entity and may be released into the blood as a number of isoforms including a ternary complex (cTnT-I-C), a binary complex (cTnT-C) as well as free forms. Immunoassay of cardiac troponin is usually designed to measure one of these forms, most commonly cTnI or cTnT. Heterogeneity and cross reactivities in many immunoassays, however, are not equimolar, complicating further the immunoassay's accuracy.[20]

Although cardiac troponin is now the established cardiac biomarker for the detection of myocardial damage,[18] its immunoassay is susceptible to interference like other immunoassays. Clinicians should therefore be aware that false positive/negative cTn results may occur. It has been suggested that in doubtful clinical situations, the use of non-immunoassay markers, such as creatine kinases[21] (CK, CK-MB), may be helpful. Because of the generally good concordance between cTn and CK/CK-MB,[22] unexplained discrepancies in the results may help to raise the possibility of immunoassay interference.

Pragmatic strategies for improving the clinical utility of immunoassay results

The technique of immunoassay was one of the most important biological innovations of the 20th century, for which the late SA Berson and RS Yalow were rightly awarded the Nobel Laureate in 1977. In numerous cases, it is simply irreplaceable. It must also be emphasised that because the vast majority of samples (about 99.5%) do not contain interfering antibodies, the majority of results obtained by immunoassays are accurate and clinical practitioners should remain confident in these tests.

Laboratories may be able to assist clinical staff by highlighting on the test report those tests that have been performed by immunoassay, distinguishing them from others less affected by interference. Immunoassay tests should be interpreted in the context of both other investigations and/or clinical correlates. If a result is in doubt and/or unexpected, then it should be discussed with the laboratory.

The clinical laboratory may repeat analysis on a fresh sample whenever feasible and if the result is confirmed, additional 'affirmative tests' may be performed. The nature of these follow-up tests will depend on the analyte under consideration. For example, interference could occur in serum hCG measurement but not in urine which is free from such interference. Repeat hCG analysis using urine rather than serum would confirm interference if discrepant.

In cases for which urine analyses may not be readily available, laboratories could perform additional 'affirmative tests' on

serum itself, such as repeat analysis using alternative immunoassay method (different reagents/different format), serial dilutions for linearity/parallelism and the use of antibody blocking agents. A discrepant result, however, in one or more of these tests could help to identify interference in a large number of samples.

A further approach is to repeat analysis on 'immunoglobulin free' serum. Highly discrepant results before and after immunoglobulin removal would be indicative of interference. Analyses in some cases could be repeated using different technology altogether such as 'chromatography-mass spectrometry', eg vitamin D, steroids, drugs.

Finally, it would be prudent practice to keep permanent records of immunoassay interference in patients' notes and/or laboratory electronicsfiles. This could help when interpreting any future immunoassay tests on these individuals.[4] It is important to emphasise that interference is totally unpredictable and can affect more than one immunoassay within the same patient. Interference can also be transmitted from mother to baby. Immunoglobulins including interfering antibodies are capable of crossing the placenta affecting immunoassay tests in the newborn, eg TSH screen for neonatal hypothyroidism.

Conclusion

Laboratory tests such as conventional biochemistry using enzymatic methods and colorimetric detection are analytically robust and clinically trustworthy. Tests carried out by immunoassays, however, are analytically more prone to errors and in some cases yield potentially clinically misleading results. Although these events are statistically low (about 0.4%), this would be of little solace for the individual concerned. Moreover, misleading results may lead to over-investigation of patients with significant financial and clinical implications.

An awareness of the limitations of these laboratory investigations is important to the user of these tests. It should trigger a more measured approach towards their utility. A clinical strategy that takes such limitations into account, coupled with good communication and dialogue between clinicians and laboratorians is highly desirable. The availability and utility of a wide array of laboratory tests for detecting and/or excluding interference from endogenous antibodies could significantly raise the accuracy and clinical efficacy of tests performed by immunoassays to levels comparable to other routine tests, thus ensuring better patient care.

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