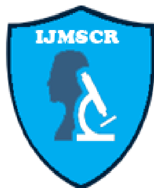

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Interferences in Diagnostic Immunoassay Procedures

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Abstract

Immunoassays are subjected to interferences with a potential to generate false increase or decrease in patient reports leading to adverse effects on patient care. Sources of such interferences are plasma, various serum proteins, heterophiles and anti-animal antibodies, drugs and cross-reacting substances. A screening protocol must be followed in order to detect the interference. If such interference goes undetected, subsequent misinterpretation or misdiagnosis could initiate irrelevant diagnostic / therapeutic procedures. Therefore a continuous dialogue must be established between the clinician, laboratory personnel and the patient in all suspected cases to avoid the harm caused by wrong diagnosis. Further research is warranted to find novel strategies in order to block immunoassay interferences.

Keywords: Immunoassay, interference, heterophiles, prozone effect, diagnosis

INTRODUCTION

Immunoassays (IA), as the name suggests utilizes the antigen-antibody immune reactions, having vast applications in the clinical laboratories including qualitative / quantitative detection of many antibodies / antigens associated with different diseases which arise due to different pathogenic viruses, bacteria or parasites. Due to selective nature of antibody binding, immunochemistry is also utilized to detect various analytes like hormones, cardiac markers, tumor markers, reproductive markers, vitamins, peptides, drugs for therapeutic monitoring, etc. and thus form part of the most frequently ordered investigations in the clinical laboratories [1]. These immunoassays utilize antibodies (monoclonal or polyclonal) generated in goat, mouse, pig, rat, rabbit, etc. [2]. Advantages of immunoassays include high sensitivity and specificity, fast turnaround time, ease of access and are economic.

Despite all the above mentioned advantages, immunoassays are subjected to interferences having potential to generate false increase (positive interference) or false decrease (negative interference) in results which have significant adverse effects on patient care [3]. Interference is the effect of substance present in the sample that alters the correct value of the result, usually expressed as concentration or activity, for an analyte. Substances that alter the measurable concentration of the analyte or alter antibody binding can potentially result in immunoassay interference.

Interference can be analyte dependent or analyte independent. Analyte dependent includes all factors that directly affect detection of analyte while analyte independent can cause signals in absence of analyte or inhibits the assay reagents directly.

Based on the site of interference in immunoassay reaction interference can falsely increase or decrease

the concentration of analyte and its magnitude depends on the concentration of that interfering substance. The change in results is not always directly proportional it can be of any magnitude.

This problem can be solved by identifying the nature of interference and its origin (exogenous or endogenous). However the origin of interference can be identified by its effect only, which has been categorized as follows:

1. Change in analyte concentration

a. Pre-analytical factors

b. Hormone binding proteins

c. Autoanalyte antibodies

d. Matrix effect

2. Change in antibody binding

a. Heterophilic antibodies

b. Human Anti Animal Antibodies (HAAA)

c. High dose hook effect

This article will summarize the most common sources of immunoassay interference and strategies for overcoming the problem.

1. Change in analyte concentration

1 (a) Pre-analytical factors: sampling and sampling type decides the degree of interference during pre-analytical phase.

Sampling: This process starts from applying tourniquet up to transfer of blood into collection tubes. In between many factors have been reported to play key roles in governing the degree of interferences during immunoassays.

Time of applying the tourniquet: Prolonged application leads to increased plasma protein concentration by approximately 5% which leads to increased ligand binding with serum and plasma [4].

Type of blood collection tube used: Blood collection tubes are not inert, they are coated with different substances like surfactants, clot activators, wall material (plastic or glass) etc. Nowadays plastic tubes are often used for convenience and easy disposal. Such plastic material from tube releases various organic substances that can interfere in immunoassays. Antigen-antibody binding is affected by lipids and silicone oils coated in tube. Besides this the water soluble silicon polymer coating of tubes have been reported to induce negative interference in the measurement of thyrotropin, prolactin and hCG and falsely elevated results in the measurement of CRP[5].

Sample type: Although serum has been preferably in use in immunoassays, plasma is also used to eliminate clotting time. Fibrinogen from incompletely clotted samples (either freshly taken or from patients with prolonged thrombin time), interfere with sampling procedure [6].If we are using serum the clot activator could produce interference. In case of plasma it's very crucial to choose proper anticoagulant from easily available choices like citrate, lithium, heparin, EDTA, fluoride/ potassium oxalate. Change in analyte concentration is observed with different anticoagulants used in cardiac troponin and various hormone assays [7].If amount of anticoagulant used is not proportionate to volume of blood, it will affect an adverse effect on the results. Besides this hemolysed, lipemic and icteric samples also produce unpredictable changes in the reported values. In case of hemolysis, proteolytic enzymes are released in the specimen; from the erythrocytes that leads to altered results for parameters like insulin, glucagon, calcitonin, PTH, ACTH and gastrin [8]. Lipemia interfere with antigen binding in immunoassays [9]. High triglycerides, cholesterol or both and esterified and non-esterified fatty acids have been reported to interfere in immunoassays for thyroxine and other endocrine assays, using second antibody and polyethylene glycol separation techniques. To avoid this interference overnight 12 hours fasting sample should be used.

Hemolysis and hyperbilirubinemia cause much less interference with immunoassays than other absorptiometric end point assays.

Time of Sample Collection: Cortisol presents with a unique diurnal cortisol cycle with an increase in secretory activity after awakening and thereafter declines for the rest of the day [10,11].

ACTH is produced and released from the basophilic cells of the anterior pituitary gland with nocturnal pulsatile secretion having highest concentration in blood in the morning. However the level drops throughout the day with a minimum concentration

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during night [12]. Testosterone, aldosterone and prolactin also behave in a similar manner [13,14,15].Therefore the time of sampling should be reported along with the patient results in order to facilitate the interpretation process.

Effect of drugs: It has been reported that steroid hormones, beta-adrenoceptor antagonists, anti-convulsants, non-steroidal anti-inflammatory drugs, heparin, etc. might interfere with thyroid function tests [16].Biotin has been reported to interfere in enzyme immunoassay of TSH and free T4 [17].

Carry over: After measuring a sample of high concentration, if the analyte is incompletely removed during washing processes; then contamination from the previous sample to the next sample could produce erroneously high patient test results in immunoassays[18].

Storage: Proper storage protocol should be followed in order to maintain the stability of analyte to avoid altered results. Serum samples subjected to continuous freezing-thawing also degrade the composition and concentration of analyte. In one study, Sgoutas D. S. and colleagues revealed the effects of increased freeze-thaw cycles and showed how the concentrations of lipoprotein (a) decreased on increasing the freeze-thaw cycles.[19] However when these samples were subjected to quick freezing at -70^{0}C and thawing, it did not produce significant decreases in Lp(a) immunoreactivity during four cycles.

Proteases present in blood samples have been reported to play a role in degrading peptide hormones such as ACTH, glucagon, gastrin, etc. To counteract these effects, protease inhibitors should be added in blood collection tubes whenever delay is expected before analysis in order to limit the degradation of analytes [20]. Thorough investigation and removal of potential influences due to pre-analytical factors on analyte concentration would assist in the proper and efficient use of an immunoassay.

I (b) **Effect of hormone binding protein**: Generally albumin, Rh-factor, complement system, lysozymes, endogenous hormone binding proteins and abnormal forms of endogenous binding proteins interfere in immunoassays [21]. Levels of such hormone binding proteins decide the extent of alteration in the measurement of the analyte. Such binding proteins may increase in any individual at any point of time due to increased synthesis, decreased clearance or may be due to congenital anomalies. One previously reported example is of low concentrations of Thyroxine-binding globulin which affects the accuracy of T3, FT3 and cortisol measurements [22]. Thus binding proteins can alter the measurable analyte concentration in the sample either by removal or blocking of the analyte [23].

1 (c) Autoanalyte antibodies: patients with autoimmune disease have circulating antibodies that may disturb the binding of analyte to reagent antibodies. A number of analyte like CK, amylase, thyroid hormones (free and total), thyroglobulin, insulin, prolactin and testosterone are affected by interference due to autoanalyte antibodies(in both IA and non-IA) [24]. Autoantibodies against thyroid hormones especially anti T4 and anti T3 antibodies are reported in Hashimoto's and Grave's disease, goitre, carcinoma, treated hyperthyroidism there antibodies interfere in total and free T3, T4 measurement. Endogenous Tg antibodies also interfere in Tg assay. Anti-prolactin autoantibodies can be present in macroprolactin form and cleared more slowly than monomeric prolactin thus accumulates in affected subjects. Normal prolactin patient will show macroprolactinemia and cause clinical mismanagement.

1 (d) Matrix effect: It is the sum of all interference effects of all components, which appear in a specimen and influence the measurement of a target analyte. Some matrix effects are derived from anti-animal antibodies, other from heterophilic antibodies from endogenous interference or from viscosity, pH value and salt concentration [25]. Labs use various proteins and surfactants and optimize their assay buffers and ionic strength to minimize the matrix effect. Samples that contain enzymes or substrates similar to those used in immunoassays may generate incorrect results. For example, a sample with elevated alkaline phosphatase may give incorrect results in assays that employ alkaline phosphatase as labels or samples containing biotin will interfere in assays employing avidin biotin as reagents.

2. Change in antibody binding (antibody interference)

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2 (a) Heterophilic antibodies: These are natural endogenous antibodies detected in the human serum and have the capability to bind immunoglobulins of other species, such as animals which are used to generate the antibodies to be incorporated as reagents for immunoassays [26]. So, when there is no history of medical treatment with animal immunoglobulins or other immunogens and the interfering antibodies have the capability to react with immunoglobulin from two or more species, such antibodies are known as heterophilic antibodies. These antibodies could interfere in immunoassays through incorrect associations between patient sera and test assays, causing a false-positive or a falsely elevated test result [27].

Since most of the investigations are being performed with fully-automated analyzers these days, there is a high probability of misdiagnosis due to interference.

It has been proved that the major effect of heterophilic antibodies is on 2-site immunometric assays using monoclonal antibodies [28]. Sandwich immunoassays use at least two antibodies directed against different epitopes of an antigen, one antibody is bound to a solid-phase, while the other is in solution and tagged with a signal moiety such as enzyme, CLIA label, etc. During the assay procedure, the antigen present in the serum or plasma binds the two antibodies. The amount of labelled antibody which gets associated with the solid-phase is proportional to the antigen concentration in the sample.

But in case when heterophilic antibodies are present in the patient's sera, it serves as the bridging agent between the two antibodies independently of antigen as explained in Fig. 1 and 2, resulting in an increase in bound labelled antibody concentration.

Figure.1. Sandwich Immunoassay

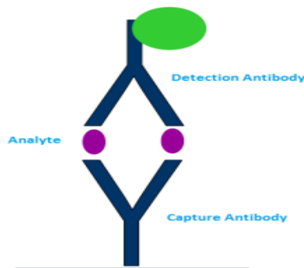
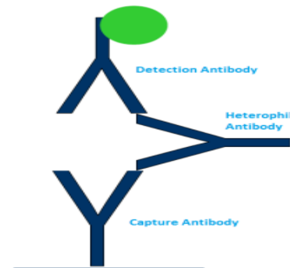


Figure.2. Interference by Heterophile Antibodies



Therefore many non-competitive laboratory assays including hormones, cardiac markers, tumor markers, infectious disease testing, etc. are subjected to interference due to heterophilic antibodies (Table.1.), having potentially drastic consequences due to misdiagnosis / falsely elevated patient reports. However heterophilic antibodies have not been observed to interfere with competitive binding assays.

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Table.1. Immunoassays affected by Heterophile Antibodies

Immunoassay		Reference
Hormones	Thyroid-stimulating hormone [29]	Hedenborg G, et al (1979)
	free thyroxine (FT4) [30]	Ghosh, et al (2008)
	Parathyroid hormone [31]	Cavalier E, et al (2008)
Tumour markers	Prostate-specific antigen [32]	Morgan B R, et al (2001)
	Carcinoembryonic antigen [33]	Kuroki M, et al (1995)
	Cancer antigen 125 [34]	Boerman OC, et al (1990)
	Alpha-fetoprotein [35]	Preissner CM, et al (2005)
	beta human chorionic gonadotropin [36]	Cole LA, et al (1999)
Other	Human immunodeficiency virus [37]	Walensky RP, et al (2001)
	C-reactive protein [38]	Benoist JF, et al. (1998)
	Digoxin[39]	Liendo C, et al (1996)
	CK MB[40]	Sosolik RC, et al (1997)
	Cardiac troponin I [41]	Fitzmaurice TF, et al (1998)

Producers of commercial immunometric assays have tried several strategies to get rid of heterophilic antibody interference e.g. (i) removal or inactivation of the interfering immunoglobulins from samples, (ii) modification of assay antibodies to make them less prone to react with heterophilic antibodies and (iii) use of buffer additives that reduce interference.

Artifactual immunoassay results caused by heterophilic antibody interference have the potential, if not suspected by the clinician or laboratory personnel, to cause inappropriate medical or surgical treatment or incorrect diagnosis that may lead to further unnecessary examinations.

2 (b) Human Anti Animal Antibodies (HAAA): These are specific polyclonal antibodies generated after contact with animal immunoglobulin. It possesses high affinity and strong binding. HAAAs responses could belong to IgG, IgA, IgM and in rare cases IgE class. If the anti-animal antibodies are elicited by animal immunoglobulins, in that case the HAAA can have anti-idiotype specificity (directed against the hypervariable region of the immunoglobulin molecule) or anti-isotype specificity(directed against the constant regions) [42].

Most common HAAAs are human anti mouse antibodies (HAMA) but antibodies to rat, rabbit, goat, sheep, cow, pig, horse can also be produced.

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Antibodies produced against mice and goat immunoglobulins are of much significance as these animals are generally used to produce commercial immunoassay reagents. Interfering endogenous antibodies should be called specific HAAAs when there is a history of medical treatment with animal immunoglobulin and immunoglobulin from the same species used in the immunoassay (e.g. human anti mouse antibodies).

Human Anti Mouse Antibodies (HAMA): Most of the immunoassay diagnostic reagents used to measure hormones, drugs and tumor markers use anti sera derived from animals. Human anti mouse antibodies are human immunoglobulins with specificity for mouse immunoglobulins. Since the introduction of in-vivo techniques using mouse monoclonal antibodies as vehicles for transporting immunoscintiraphic or chemotherapeutic agents to tumor sites, the presence of HAMA has become a major problem [43]. The concentration of HAMA in the plasma of patients treated in this way is several folds greater than that of heterophilic antibodies. HAMA interference has been reported for many analytes like cardiac markers, drugs and tumor marker, thyroid function test etc. Two site immunoassays are more prone to interference from antibodies to animal IgG in human serum and may cross react with reagent antibodies especially those from the same species. Methods that use one mouse monoclonal antibody in IA are less prone to interference from HAMA. HAMA bridging interference produces artificially higher results because HAMAs bind to and immobilizes mouse antibodies in place of substrate. Secondary labelled antibodies will then bind to HAMA and produces a positive signal falsely indicative of substrate presence.

2 (c) High dose hook effect: The one-step sandwich immunoassay is increasingly replacing the traditional two-step immunoassay as it is preferred by most high throughput systems to increase speed. Unfortunately, the one-step sandwich immunoassay suffers from the hook effect irrespective of the analyte characteristics. The "high-dose hook effect" or "prozone phenomenon" is characterized by the paradoxical fall in the dose-response curve in the high-dose region leading to absurd low results in samples that have extraordinarily high concentrations of antigen [44].

Figure 3(a) and 3(b) explains the saturation curve of antibody with antigen and Hook effect respectively. It is a specific type of interference found only in one step sandwich assays with very high analyte concentrations and was first reported in 1974 by Miles LE, et al in a two-site immunometric assay[45].

An example of "hook effect" in prolactinomas was presented by Frieze TW, et al in 2002. In the initial laboratory testing of a 65year old man with a giant prolactinoma, the prolactin level of 164.5 ng/mL (normal range: 1.6 to 18.8) was obtained. The results of tissue staining during pathological examination of specimen received during the surgical debulking procedure were consistent with prolactinoma. Dilution testing (serial dilutions) of the original serum prolactin sample revealed a prolactin level of 26,000 ng/mL which confirmed the occurrence of hook effect in prolactin immunoassay [46]. In all such cases, the reduced assay signal is caused by excessively high concentrations of the analyte simultaneously binding both capture and detecting antibodies. This prevents the formation of the required complexes with capture antibody, analyte and detecting antibodies.

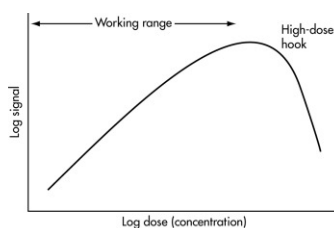

Figure 3(a) Hook Effect

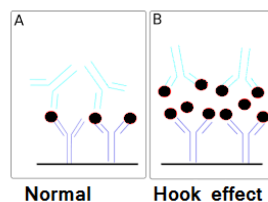

Figure 3(b) Hook Effect

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Therefore, in immunoassays with very wide measurement ranges and high analyte concentration, antigen excess results in false low values e.g. calcitonin [47], PSA[48], CA 125[49], AFP[50], myoglobin[51], prolactin[46], etc.(Table 2).

TABLE 2. Examples showing Hook Effect or Prozone Effect

Assay	Hooked Value	True Value	Study
Calcitonin	48 ng/liter	662 ng/liter	Leboeuf R, et al (2006)
PSA	106 µg/liter	1020 µg/liter	Vaidya HC, et al (1988)
CA 125	< 500 kAU/liter	9500 kAU/liter	Pesce MA, et al (1993)
AFP	525 kU/liter	2, 461, 400 kU/liter	Jassam N, et al (2006)
Myoglobin	1000 µg/liter	> 395,000 µg/liter	Kurt-Mangold M, et al (2012)
Prolactin	164.5 ng/mL	26,000 ng/mL	Frieze TW, et al (2002)

Testing for the prozone effect requires performing the test with serial dilutions of the sample (1/20, 1/50, 1/100) and checking the linearity [52].The specimen should be diluted till the results of two different dilutions match (taking into consideration the dilution factor). Prozone effect is confirmed, if in diluted samples, higher values are measured than in non-diluted sample. However, this approach results in increased reagent costs for assays that may only rarely encounter extremely high analyte concentrations. An alternative approach involves pooling of patient samples and measuring the pool and a 10-fold dilution of the pool [53]. In case when one or more of the samples from the pool is falsely low due to the prozone effect, then the values obtained from the undiluted and diluted pools (after correcting for the 10-fold dilution) will differ significantly.

How to deal with suspected interference:

Before removing interfering substance, it is essential to initially identify the presence of interference. During follow-ups the clinicians were the first to know whether the patient values correlates with the clinical picture of the disease or not. If discordant results are found, the physician is expected to inform the laboratorian about the suspected results and the patient's history in terms of medication, immunization, nutritional supplements, etc. must be shared. The patient should also be queried for any possibilities of close contact with animals or history of past treatment with animal immunoglobulins. A screening protocol must be followed in order to detect the interference. Clinical picture must not be underestimated in comparison to laboratory results. Any interference detected during laboratory procedures must be properly documented in the patient's records for future use. Besides this immunoassay reagent manufacturers must reveal the possible interferences in their kits.

The approach to eradicate the interference depends on the type of interference present in the specimen. The most common steps to be applied in order to check the suspected samples are as follows:

Confirmation of results by other immunoassay methods:

Results from suspected samples could be crosschecked for the presence interference by using a different method or by an IA of different origin using different antibodies (if available in the laboratory). Alternatively samples could be sent to other laboratories to detect the suspected interference using other immunoassay platforms. However if similar results are obtained by all other methods, it indicates that identical interfering mechanism prevails in all the methods applied to remove the interference.

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By dilution and recovery studies:

This is the most common practice that suspected sample is serially diluted and is checked for linearity in results. Sometimes satisfactory linearity and/or recovery may be observed even in the presence of an interfering substance. As well as some specimens that are apparently free from interfering substances do not dilute out linearly, particularly in some of the more complex tumour marker assays (e.g. CA19-9) [52]. Detection of endogenous antibody interference could be easily done by running the sample with serial dilutions and by getting a nonlinear response. However nonlinear responses in dilution studies could also originate due to the heterogeneous nature of the analyte [54]. If the result on dilution is higher than for the undiluted sample, then the undiluted sample most likely exhibited the prozone effect [55].

Use of heterophilic blocking reagents:

Nowadays heterophilic antibody blocking tubes are commercially available which can be used as per the instruction of manufacturers [56].

Addition of non-immune animal serum:

Adding non-specific immunoglobulins to the reaction mixture may reduce interference if the human antibodies bind to these instead of binding the assay antibodies. Murine and bovine antibodies reduce interference in the highest percentage of patient's samples and also have the highest avidity for heterophilic antibodies [57].

Polyethylene glycol precipitation:

Polyethylene glycol (PEG) could be used to precipitate potentially interfering antibodies and other high molecular weight complexes [58].

Sample extraction:

In case of suspected interference in steroid immunoassays (e.g. Testosterone), extracting the specimen with diethyl ether, re-suspending in appropriate analyte-free diluent and re-assaying may be used. The extraction step separates the steroid from any binding proteins as well as removes water-soluble steroid conjugates [59].

In more complex cases we can use:

Gel filtration chromatography, Immunoadsorption chromatography on immobilized IgG binding proteins or double radial immunodiffusion (Ouchterlony) studies

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