Resolving discordant samples in clinical laboratory practice

By Pradip Datta, PhD, DABCC

The growing importance of the clinical laboratory in patient healthcare is evident from the fact that about 70% of medical decisions are now based on laboratory test results. The role of the clinical laboratory, aided by advances in analytical science and increased automation, has become even more prominent, as cost pressures have reduced patient stays in the hospital. Laboratory results — normal or abnormal — help screening, diagnosis, indication of therapeutic pathways, and monitoring of therapy. But a variety of causes, pre-analytical or method-dependent, may lead to inaccurate “discordant” results; such results may lead to incorrect diagnosis and therapy, risking increased morbidity or mortality. What causes lead to false-positive or false-negative results, especially in immunoassays and chemistry tests?

Errors in a test result may belong to three classes: random, systematic, and sporadic.

- **Random error** causes poor precision; however, automation and improvement in analytical science are advancing precision.
- **Systematic errors** are caused by incorrect calibration or differences in standardization of the test. Often, when a patient moves from one hospital to another, some lab results change because different methods may be used in those labs. Government regulatory authorities and international professional organizations have worked together to produce reference and survey materials, thus reducing the systematic error.
- **The sporadic error**, however, which occurs in some specimens but not others, is the largest cause of discordant results.

### Causes of discordant results

What are the causes that produce discordant results in immunoassays? Using highly specific antibodies and binding proteins, these assays can measure very small concentrations (as little as femtomolar or $10^{-15}$ M level) of clinically important analytes in a specimen. Erroneous immunoassay results may be caused by two major sources: antibody specificity and assay interferents. While antibodies are very specific, they may recognize other molecules having a molecular “signature” similar to the analyte. Depending on the assay architecture, such molecules, called cross-reactants, may increase or decrease the assay signal, thereby falsely increasing or lowering the test results. Such cross-reactants could be metabolites of the analyte with varying amounts of physiological activity, a coadministered drug or nutrition aid, or other endogenous component of the sample. Ideally, the cross-reactivity of the metabolites in an immunoassay should parallel their physiological activities. An example of this concept is a chemiluminescent immunoassay (CLIA) for the cardiac drug, digoxin, presented in Table 1. The effect of cross-reactivity depends on the cross-reactant and analyte concentrations in the specimen, as shown in Figure 1. Examples of endogenous cross-reactants include similar classes of compounds, like steroids and gonadotrophins, interfering in one another’s assays. Thus, human chorionic gonadotrophin (hCG) often falsely increases results of other hormones with similar structures (e.g., leutrophin, LH, or thyroid-stimulating hormone, TSH) in some assays. Since hCG concentrations greatly increase during pregnancy, even a very small cross-reactivity of the assay antibodies to hCG results in large error.

Another example is the interference of endogenous digoxin-like immunoreactive factors (DLIF) in digoxin immunoassays. DLIF concentration increases in certain groups of patients, like neonates, pregnant women, and patients with liver and renal diseases. While it is easier to find out if a coadministered drug generates a discordant result, it is much more difficult to find out if the interferent is one or more components of a herbal medicine (e.g., Chinese medicines like ginseng, Chan Su, and Dan Shen). Hemolysis, icterus, and turbidity from lipids are common sources of assay interference, and most kit manufacturers list them in the kit.

![Table 1. Cross-reactivity of the major digoxin metabolites in a commercial chemiluminescent immunoassay (CLIA) and an experimental radio-receptor assay (RRA) for digoxin. The RRA used the actual digoxin receptor from human heart muscles (instead of an antibody). The CLIA used an antibody for digoxin, screened to match the metabolite cross-reactivity. The CLIA does have metabolite cross-reactivity comparable to the metabolites’ cardioactivity and RRA.](image-url)
Forms and formats contribute to discordance

Another source of assay discordance is the heterogeneity of the analyte, especially for the peptide analytes. Additionally, the analyte may be present in the specimen as free, as well as bound to other proteins. Thus, the prostate-cancer marker, prostate-specific antigen (PSA), stays in serum as both a free form and bound to serum proteins like α1-antichymotrypsin and α1-macroglobulin, or the cardiac marker troponin-I (cTnI) may exist as free or complexed with troponins C and T. If two assays recognize these forms differently, they will report dissimilar results for the same sample. Table 2 shows that five commercial cTnI assays have different responses to the free vs. complexed (with troponin C) forms of the analyte. Prolactin exists in serum as a monomer, dimer, trimer, and polymer. Since these forms react differently to different antibodies, discordance has been observed among different prolactin immunoassays.

Some discordant results may arise from the particular immunoassay format used. For example, in a two-site immunometric assay, which uses two separate antibodies to the analyte to form a “sandwich” complex, the concentration of the complex is directly proportional to that of the analyte. In such an assay, if the antigen concentration is very high, separate analyte molecules may bind the two separate antibodies, thus reducing the complex formation, and thereby falsely lowering the test results. Such examples of “hook” or “prozone” effects are found in assays measuring a wide range of the analytes, (e.g., hCG and PSA); based on reagent formulations, the hook analyte concentrations are different in different assays.

Other culprits in discordance

Some pre-analytical factors also cause discordant results. Most important among them is the type of specimen-collection tube used, and how the specimen is stored. Thus, if the analyte’s or assay’s reagents are affected by the various additives found in sample-collection tubes, different results will be obtained for the same patient depending on the type of collection tube. An example is cTnI assays, where Ca²⁺ ions are needed to maintain the complexed forms of the analyte. Since anticoagulants like EDTA or heparin bind Ca²⁺ ions, some cTnI assays show different results for serum and different types of plasma. If an assay uses an enzyme to generate the signal, (e.g., alkaline phosphatase or horse-radish peroxidase), the presence of the similar enzymes or substances that affect the enzyme activities in the sample or reagents may affect the reported result.

Some analytes are very sensitive to storage conditions. Thus, plasma samples for ammonia measurement must be analyzed as soon as possible after collection and should be kept refrigerated until analysis, minimizing atmospheric exposure. Free PSA and parathyroid hormone are also unstable and must be stored refrigerated. While automated analyzers have greatly improved lab productivity and increased the quality of results, they also bring some system-specific issues interfering with assay results (e.g., sample or reagent probe carryover, goodness of fit with the calibration curve algorithm, and reaction cuvette carryover). Assay manufacturers, however, take utmost steps to minimize such interferences.

One of the most common assay interferences found in the clinical lab is from antibodies in the specimen. While most such interference is from endogenous antibody (the most common being the ‘heterophilic’ antibody and autoantibody), therapeutic antibody may also cause assay interference. An example of the latter is Digibind, which consists of Fab-fragments of digoxin antibody, administered to neutralize digitalis toxicity. Digibind, especially in high concentration in a sample, causes false-positive interference in many digoxin immunoassays. Ultrafiltration is a simple experiment to resolve such discordance; the specimen is ultrafiltered through a membrane with a molecular weight cutoff range of 10 kDa. Most protein interferents, as well as those which are highly bound to proteins (e.g., DLIIF), are retained while the relatively less-protein-bound analyte, digoxin, collects in the filtrate and is analyzed.

Autoantibodies are antibodies that exist in a patient, binding preferentially to the analyte of choice (or components of the reagents used in the assay). Such interference is often found in thyroid assays. Autoantibodies to some assay reagent components (e.g., antiavidin or antienzyme) may yield incorrect results for reagents using avidin or those enzymes. Rheumatoid factors, another type of autoantibody, also can interfere in immunoassays. Heterophilic antibody interference is a major source of discordant results reported in the literature. These antibodies interact with the assay antibodies (raised mostly in mice, goat, sheep, or rabbits) used in the reagents. The heterophilic antibodies may form spontaneously or in response to close contact with the animals or antibodies used earlier in their therapy.
sample should be confirmed by repeating the assay(s) to rule out instrument error. If possible, a third “referee” assay may be run to learn which result is correct. Dilution studies often confirm and resolve discordance. If heterophilic interference is suspected as the cause of discordance, the sample may be incubated with blocking reagents and repeated in the assay.

While laboratory tests are increasingly used to aid earlier and faster diagnosis — in turn, improving healthcare and reducing costs — laboratorians must constantly be on watch to detect and resolve discordant results.

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References

or diagnosis. In most of the affected immunoassays, heterophilic antibodies interact with the two analyte-specific reagent antibodies, forming a “pseudo-complex” that falsely raises the test result.41 Human antimuscle antibody (HAMA) interference is a special case of heterophilic antibody interference, where the HAMA interacts with murine antibodies. Assay manufacturers often add agents to block heterophilic antibodies; derived primarily from nonspecific animal antibodies, the blockers bind to the sample’s interfering antibodies, neutralizing them before they can interact with the analyte-specific antibodies.42 This, however, is not a foolproof method, because the blockers can neutralize only up to certain concentrations of interfering antibodies. If the specimen has a very high concentration of the heterophilic antibodies, these can saturate out the blocking reagent and then affect the assay.

Resolving discordant results

How can discordant samples be identified among the large number of samples that a typical laboratory processes? While it is easier to detect such discordance with abnormal results (because they are flagged by the auto analyzers), the discordant result that falls in the normal range is the most difficult to detect. This is where the skill and experience of the laboratory helps. Patient history also helps: Does an analyte result match with other results from the lab? Does the patient chart alert of any discordant results found earlier? Is the patient taking any medication that is known to interfere with some assays? Of course, spotting a discordant result is easier if two different assays with the same specimen report very different results (e.g., when method comparison is done in evaluating a new assay). Once identified, a discordant