Immunoassay interferences and their impact on patient care

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Focus 2019 - Glasgow
I, Carmen L. Wiley, have a financial interest/arrangement or affiliation with one or more organizations that could be perceived as a real or apparent conflict of interest in the context of the subject of this presentation, they are:

- Affiliation/Financial Interest: VERAVAS, Inc. – Chief Clinical Officer
- Stock/Shareholder: VERAVAS, Inc.

However, I will not be discussing any VERAVAS products today and no products are currently available.
LEARNING OBJECTIVES

After completing this activity, the learner will be able to:

1. List the common interferences that impact immunoassay results
2. Describe how interferences impact patient results
3. Create a plan for mitigating these interferences
Every 9 minutes, someone dies due to an incorrect or delayed medical diagnosis.

70% of physician decisions are influenced by diagnostic testing.

https://www.improvediagnosis.org
http://jalm.aaccjnls.org/content/1/4/410
2% of lab results are incorrect
Half of incorrect results can lead to incorrect treatment

Physicians rely on diagnostic tests
Inaccurate results can be caused by multiple interferences in blood or urine (e.g., Heterophilic Interference and Biotin in Immunoassays)

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1904417/
Dirty laundry analogy

The problem:

Didn’t know the RED sock was there until it was too late

Sample Interference

Today’s solution:

Knew a RED sock could be there, tried to mitigate the possibility with a color catcher sheet – doesn’t always work

Assay Design and Blocking
PROBLEM
False Test results due to sample interferences that impact immunoassay signal response

http://scantibodies.com/hbr/#hetero
False test results

The clinical impact

False Positives
Defined as results greater than the reference range, clinical cut-off, or medical decision point

False Negatives
Defined as results below the reference range, clinical cut-off, medical decision point, limit or detection, or limit of quantitation

False Normals
Defined as results within the reference range or reference interval
False Positives
Defined as results greater than the reference range, clinical cut-off, or medical decision point

- Unnecessary interventional therapy
- Unnecessary hospital admission
- Unnecessary risk associated with hospitalization
- Unnecessary emotional stress
- Unnecessary follow up testing
- Increased length of stay in the Emergency Room (ER)
- Death
False test results

The clinical impact

False Negatives

Defined as results below the reference range, clinical cut-off, medical decision point, limit or detection, or limit of quantitation

- Adverse event outside hospital or ER setting, including death
- Missed opportunity for early intervention
- Rehospitalization
- In the case of Infectious disease, can lead to outbreak or spreading disease to others
- Can put blood donor supply at risk
- May result in discontinued or modification of necessary medical treatment/drugs
- False sense of security
False test results

The clinical impact

False Normals
Defined as results within the reference range or reference interval

- Adverse event outside hospital or ER setting, including death
- Missed opportunity for early intervention
- Rehospitalization
- In the case of Infectious disease, can lead to outbreak or spreading disease to others
- Can put blood donor supply at risk
- May result in discontinued or modification of necessary medical treatment/drugs
- May confuse Physicians/Clinicians
22 influential laboratories in the US were surveyed

Most labs state that they don’t know they have a problem until they receive a call from a clinician inquiring about a test

20 of the 22 people interviewed thought interferences were a problem

Investigating Interferences

Most lab directors indicated that they are responsible for the troubleshooting and reporting of the problem

Technologists perform the bench portion of the troubleshooting

Lab directors manage the medically based investigation including gathering patient information and communicating with the clinician

*Study conducted by IOI on behalf of VERAVAS, Inc. December 2018 Final Report.
Incidence of interferences due to heterophilic antibodies is between 0.05 - 80% depending on the analyte being measured.

6 main interferences affecting measurements of TSH are reviewed. Prevalence of some of these conditions has been reported to approach 1%.

Clinical impact of thyroid interference on immunoassays. At least 50% of documented thyroid interferences led to misdiagnosis and/or inappropriate management of the patients (150 subjects reviewed).
Mayo Survey\textsuperscript{1,2}

4,000 Mayo Clinic outpatients were surveyed

1,944 returned completed paper questionnaires (972 female, 963 male, nine unspecified)

8\% said, yes, they were taking biotin

1,442 plasma samples studied from Emergency Room patients

Nearly 50\% had detectable biotin concentrations

7.4\% had biotin concentrations $\geq 10$ ng/mL

2\% percent had biotin concentrations $\geq 20$ ng/mL.


INTERFERENCE IN CLINICAL ASSAYS

Interference has been reported in numerous clinically and commercially important immunoassays such as:

- Cardiac Troponin and myoglobin
- 25-OH vitamin D, total
- Human chorionic gonadotropin (hCG and beta-hCG)
- Serum follicle-stimulating hormone (FSH)
- Rheumatoid arthritis
- Free triiodothyronine (FT3) and free thyroxine (FT4)
- TSH
- Antiphospholipid antibody assays (cardiolipin, B2-glycoprotein)
- Coagulation assays (lupus anticoagulants)
- Tumor marker assays
- Drug monitoring (tacrolimus) and therapeutic product assays

TYPES OF INTERFERENCE

There are many sources of sample specific interference in the clinical laboratory such as:

- Sample type (i.e. whole blood, plasma, serum, stool, urine)
- Carry-over
- Freeze/thaw
- Stability (sample storage)
- Hemolysis
- Icterus
- Lipemia
- Effects of anticoagulants
- Binding proteins
- Drugs and drug metabolites (i.e. biotin)
- Cross-reactivity
A TROUBLESOME INTERFERENCE

Heterophilic antibody interference such as:
- human anti-animal antibody (HAAA)
- human anti-mouse antibody (HAMA)
- rheumatoid factor and other autoantibodies

are problematic as they are difficult to detect and can drastically affect patient management.

Studies have been completed to try and determine the prevalence of heterophilic antibody interference, and results range from as low as 0.05% to as high as 80% for a given assay and patient population.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1904417/
DEFINITION OF A HETEROVIPHILIC INTERFERENCE

The definition of heterophilic, HAMAs and human anti-animal antibodies (HAAAs) is imprecisely employed in the literature (and by me)

- HAAAs are monospecific, high-affinity antibodies directed against animal epitopes from goats, rabbits, sheep, horses or, more frequently, mice (HAMA)
- Heterophilic antibodies are weak polyspecific antibodies (usually of low titer) formed early in the immune response prior to affinity maturation. They typically react with immunoglobulins derived from at least two species.
- Rheumatoid factor (RF) also belongs to this category as it reacts against the Fc region of human immunoglobulins, displaying cross-reactivity against animal antibodies
- In daily laboratory practice, the term “heterophilic antibody” is typically used whenever one suspects that a patient’s sample contains antibodies that cause false results by binding to the assay antibodies

In this talk I will consider true heterophilic antibodies and HAAAs together, since they may cause similar types of assay interference, but I’ll likely call them HAMA, because that’s easier to say than HAAA.
COMMON IMMUNOASSAY FORMATS USED TODAY

• Double antibody sandwich assays
  – The amount of antigen present in the patient sample is directly proportional to the amount of relative light units (RLU) or signal detected by the system

• Competitive inhibition assays
  – An inverse relationship exists between the amount of antigen present in the patient sample and the amount of RLU or signal detected by the system

• Delayed capture assays
  – The capture antibody is labeled with a tag (i.e. biotin, fluorescein) for subsequent capture by an anti-tag solid phase
  – Assay signal is dependent on successful capture of the tag-labeled capture antibody by the anti-tag solid phase
DOUBLE ANTIBODY SANDWICH ASSAY FORMATS

A. The conjugate binds antigen first followed by capture antibody
B. The capture antibody binds antigen first followed by conjugate
C. Both the conjugate and capture antibody bind antigen at the same time
COMMON IMMUNOASSAY FORMATS USED TODAY

• Double antibody sandwich assays
  – The amount of antigen present in the patient sample is directly proportional to the amount of relative light units (RLU) or signal detected by the system

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COMPETITIVE INHIBITION ASSAY FORMATS

A. Conjugate and antigen compete for capture antibody (true competition)
B. Antigen binds to capture antibody in the first incubation, and a molar excess of conjugate is added in the second incubation to bind to capture antibody (backfill or piggyback)
COMMON IMMUNOASSAY FORMATS USED TODAY

• **Double antibody sandwich assays**
  – The amount of antigen present in the patient sample is directly proportional to the amount of relative light units (RLU) or signal detected by the system.

• **Competitive inhibition assays**
  – An inverse relationship exists between the amount of antigen present in the patient sample and the amount of RLU or signal detected by the system.

• **Delayed capture assays**
  – The capture antibody is labeled with a tag (i.e. biotin, fluorescein) for subsequent capture by an anti-tag solid phase.
  – Assay signal is dependent on successful capture of the tag-labeled capture antibody by the anti-tag solid phase.
A. The conjugate and capture antibody bind antigen and form a solution-based sandwich, and a SAv coated solid phase binds to the biotin tag on the capture antibody
B. Capture antibody binds antigen, a SAv coated solid phase binds to the biotin tag on the capture antibody, and Conjugate forms a sandwich with the capture antibody-antigen complex
Reminder, true heterophilic antibodies and HAAAs will be considered together, since they may cause similar types of assay interference.

In the next few slides they are labeled as HAAA, but I’ll likely call them HAMA, because that’s easier to say than HAAA.
HAAA STERIC HINDRANCE IN SANDWICH ASSAY

A. HAAA IgM binds capture antibody and interferes with antigen capture and conjugate binding to the solid phase.
B. HAAA IgM binds conjugate and interferes with conjugate antigen binding as well as binding to the solid phase.
C. Both conjugate and capture antibody bind antigen but HAAA IgG interferes with subsequent sandwich formation.
**HAAA STERIC HINDRANCE IN COMPETITIVE ASSAY**

A. HAAA IgM binds capture antibody and interferes with conjugate binding to the solid phase.

B. HAAA IgM binds capture antibody during the first assay incubation and interferes with the conjugate binding during the second assay incubation.
**HAAA BRIDGING IN SANDWICH ASSAY**

A. HAAA IgM binds Fab or species specific epitope(s) on both capture antibody and conjugate resulting in excess conjugate binding.

B. HAAA IgM binds the Fc portion of conjugate and results in excess conjugate binding.

C. HAAA IgM binds the Fc portion of both capture antibody and conjugate and results in excess conjugate binding.
A. HAAA IgM binds conjugate bound to the capture antibody as well as additional conjugate.
B. HAAA IgG binds conjugate bound to the capture antibody in the back fill assay incubation as well as additional conjugate.
OTHER INTERFERENCE THAT IMPACT IMMUNOASSAYS

- Over the counter (OTC) supplements
- Vitamins
- Herbal remedies
- Therapeutically prescribed drugs

These are all things people ingest.
BIOTIN AS AN EXAMPLE INTERFERENCE FOR THINGS PEOPLE INGEST

A. Free biotin binds to SAv biotin binding sites and competes for binding of the biotin-labeled capture antibody and sandwich complex

B. Free biotin binds to SAv biotin binding sites and competes for binding of the biotin-labeled capture antibody prior to the final assay incubation and conjugate addition
CURRENT STRATEGIES TO TROUBLESHOOT INTERFERENCE

Laboratories can troubleshoot and confirm suspected interference in patient specimens using approaches such as:

- Review the manufactures Package Insert – understand assay specific interferences
- Dilution recovery and dilution linearity
- Protein A or protein G affinity chromatography
- Polyethylene glycol (PEG) protein precipitation
- Acetonitrile (organic solvent) protein precipitation
- Immunosubtraction (column-based affinity absorption such as streptavidin-agarose absorption)
- Size exclusion chromatography
- Pre-incubating the specimen with mouse, goat and/or bovine IgG
- Scantibodies HBR tubes
- Testing the specimen by an alternative assay or technology

ASSAY DESIGN APPROACHES TO REDUCE INTERFERENCE

• The use of antibody Fab fragments, antibodies from different subclasses and species, chimeric antibodies, aptimers and new blockers

• Blocking strategies and reagents used today include:
  – Protein and antibody based blockers (i.e. BSA)
  – Polymeric blockers (i.e. Polyvinylalcohol (PVA) or polyvinylpyrrolidone (PVP))
  – Surfactants and detergents (i.e. Tween 20, polyethylene glycol (PEG)-based blockers)
  – Commercial blockers (i.e. Scantibodies, HBR, IIR, Meridian Life Science TRU Block..)
  – Inactivated assay components (i.e. inactivated ALP)
Other approaches used today to reduce assay interference include:

- Addition of blocking reagents to the conjugate, beads, and/or assay buffer
- Reduced sample size
- Order of addition
- Delayed addition
- Washes (i.e. 1-step vs. 2-step)
- Incubation time with blocker(s)
- Use of Assay Buffer
ASSAY BLOCKING TODAY

• Legacy assays – often aren’t using the approaches described
• New assays – are the best opportunity to incorporate the approaches described
• Improved assays – are the other opportunity to add these approaches
  – can be difficult because requires major redesign
  – requires revalidation and regulatory submission
• No such thing as a perfect assay
SO THIS BRINGS US BACK TO THE PROBLEM OF ASSAY INTERFERENCE

While it is known that immunoassays are susceptible to interference, the clinical laboratory may still report erroneous results if:

- results are not recognized and flagged by the instrument (analyzer) or laboratory
- if the physician does not notify the laboratory that the patient result does not fit the clinical picture

SO WHAT IF.....
DIRTY LAUNDRY ANALOGY

The problem:

Didn’t know the RED sock was there until it was too late

Sample Interference

Today’s solution:

Knew a RED sock could be there, tried to mitigate the possibility with a color catcher sheet – doesn’t always work

Assay Design and Blocking

Tomorrow’s solution:

If there is a RED sock, we know it is there and we remove it before washing.

An Ideal Solution
I hope you are now able to:

1. List the common interferences that impact immunoassay results
2. Describe how interferences impact patient results
3. Create a plan for mitigating these interferences
THANK YOU

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