

Selected Issues in Immunoassays: How to Evaluate Problems and support Clinically Relevant Decisions

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Generally Issues are Related to

■ Assay/Method

- RIA vs Others (ELISA, EIA, CLIA, FPIA, IRMA, TIA)
- RIA... Extraction (Indirect) vs Non-Extraction (Direct)
 - » Cortisol, Vitamin D, Testosterone, Progesterone, etc.
 - » Well Documented... Clin Chem 49:1381 – 1395 (2003); EQAS & CAP Summary Reports
 - » Method of choice now is HPLC/Tandem Mass Spectrometry

Generally Issues contd 1...

■ Analyte

- Free & Bio-available Testosterone; Free & Complexed PSA; Beta hCG's; Digoxin & Digoxin-Like Substances
- Well Documented.... Dr P Y Wong; Manuf. Kit Inserts; Clin Biochem

■ Endogenous & Exogenous Interferents

- Drugs (e.g. Digoxin & anti-aldosterone/hypertensive agents)
- Immunoglobulins (e.g. Macroprolactin; Macro-Alk Phos)
- Monoclonal Proteins - TIA's

■ Others

- Antisera Specificity & Source/Type; Polyclonal vs Monoclonal; Single vs Double Antibody; Human; Animals

Generally Issues contd 2...

- Good reviews on analyte related issues
 - » Dr P Y Wong. Irritating Interference and Erroneous Errors in Hormone immunoassays... a view from the laboratory . Presented at the OSCC Annual Scientific Meeting , Hamilton Chamber of Commerce, Nov 2003.
 - » Dr L A Cole. Immunoassay of human chorionic gonadotropin, its free subunits, and metabolites. Clin Chem 43: 2233 – 2243 (1997)

RIA vs Others – Anti-ds DNA

RIA vs. EIA A
(n = 83)

	RIA Normal (Up to 3.6 KU/L)	RIA Abnormal (> 4.1 KU/L)
EIA A Normal (< 25 IU/ml)	51	11
EIA A Abnormal (> 35 IU/ml)	5	16

Overall Agreement (%): 81
False Positive Rate (%): 9
False Negative Rate (%): 41

RIA vs Others – Anti-ds DNA

RIA vs. EIA B (N = 62)

	RIA Normal (Up to 3.6 KU/L)	RIA Abnormal (> 4.1 KU/L)
EIA B Normal (< 25 IU/ml)	42	10
EIA B Abnormal (> 35 IU/ml)	2	8

Overall Agreement (%): 81

False Positive Rate (%): 5

False Negative Rate (%): 56

RIA vs Others – Anti-ds DNA

EIA A vs. EIA B
(n = 57)

	EIA A Normal (< 25 IU/ml)	EIA A Abnormal (> 35 IU/ml)
EIA B Normal (< 25 IU/ml)	43	4
EIA B Abnormal (> 35 IU/ml)	3	7

Overall Agreement (%): 88
False Positive Rate (%): 7
False Negative Rate (%): 36

Overall Assessment; Anti-ds DNA: EIA/RIA vs IFA (Gold STD)

		ANA IFA Pattern	
		- (Homogenous)	+ (Homogenous)
RIA	-	17	8
	+	12	9
EIA A	-	22	9
	+	6	6
EIA B	-	15	5
	+	3	1

	RIA	EIA A	EIA B
% Agreement (overall)	57	65	67
False Negative Rate (%)	41	21	17
False Positive Rate (%)	47	60	83

Analyte Related – Beta hCG

- Antibody Hetero-specificity to different hCG species:
 - Intact Beta hCG assay
 - Free hCG Beta-subunit assay
 - Total hCG assay
 - Total hCG + Beta assay
- False positive hCG results leading to unnecessary surgery and chemotherapy and needless occurrences of diabetes and coma

Analyte Related – A case of Anti-analyte antibodies (PY Wong, OSCC Nov 2003)

- 84 y old female with clinically diagnosed hypothyroidism
- Initial Lab Result: TT4 >500 nmol/L(RI:60 – 150)
- Further TFT's:
 - TT4 = 840 (after methanol Extraction = 140)
 - TT3 = 50 (after methanol extraction = 20; RI 1.2 – 2.8)
 - FT4 (analog) = 124 nmol/L (RI: 10 – 25)
 - FT4 (dialysis Equ) = 10 (RI: 12 – 42)
 - TSH = 18 (0.3 – 5.0)
- Clinical Protocol
 - If TSH, FT4, TT4, TT3 all elevated, refer patient for MRI of the brain to rule out pituitary adenoma
 - MRI result: No evidence of Pit Adenoma
- Appropriate Diagnosis: Hypothyroidism (high TSH) with artifactual elevated thyroid hormone concentrations due to anti-T4 & anti-T3 antibodies. Even after methanol extraction TT3 & TT4 remained significantly elevated.

Analyte Related – Testosterone

- Immunoassays (esp direct) not suitable for assaying Testo in children & woman; even those with solvent extraction may variable results – HPLC & Mass Spec method of choice
- Taieb et al Clin Chem 2003; 49(8): 1381 – 1391
 - 10 Immunoassays vs ID/GCMS for total Testo
 - 7/10 had higher values than GCMS (up to 46% higher) in Females
 - 4/10 had lower values than GCMS (up to 46% lower in Males
 - Magnitude of Mean Differences (Bias) were also different in males & females
 - 0/10 was sufficiently reliable for use in Children & women
 - 7/10 had $r > 0.95$ but only in men; $r < 0.95$ for females in all 10.
- Same for Free Testo - Yet several labs in Ontario are currently using the DPC RIA assay
- Calculated Free Testo & BAT
 - Produce comparable, if not superior, results to the measured parameters
 - Issue is how to implement the new & more accurate alternatives into the routine laboratory that relies heavily on automation

Endogenous/Exogenous Interferents – Drug Effects on Digoxin Assays

- Digoxin Assays: Frequent, Substantial, and Potentially Dangerous Interference by Spironolactone, Canrenone, and Other Steroids
 - Werner Steimer, Christine Muller, and Barbara Eber. Drug Monit & Toxicol 48: 507–516 (2002)
 - 9 assays (Major Manufacturers – Abbot, Dade Behring, Roche & Ortho)
 - EMIT, MEIA, FPIA, TIA, ECLIA
 - DPC's CLIA Assays were **Not** studied
- **Conclusion**
 - **Vitros, TIA & EMIT: Minimal or No Interference**
 - **TDx, ACA & ECLIA: Falsely Increased Digoxin**
 - **Dimension, AxSYM & Imx: Falsely Reduced Results**
- **False-Negative & False-Positive Effects were observed at relatively low drug concentration**

Endogenous/Exogenous Interferents – Immunoglobins

- Macroprolactin (Prolactin-Ig Complex)
 - Falsely Increased Prolactin Levels; Suggesting Prolactinoma
 - MPRL; not biological active
 - Most, but not all, commonly used methods are susceptible
 - Need to be delineated from true prolactinemia
 - If unidentified, can lead to unnecessary clinical work-up
- Common Approaches
 - Screen with existing methods & send elevated PRL's & Ref out to other testing site – Issue is TAT & difference in methodology for PRL
 - Removal of macroPRL on elevated PRL & repeat testing
 - » Ultracentrifugation
 - » PEG precipitation
 - » Gel Filtration chromatography (GFc; gold std but not amenable to run use)
 - Issues are effectiveness, ease-of-use and cost of the separation techniques, as well as, appropriateness of cut-off points and interpretation of results
 - A Case of MacroPRL method comparison (GDML)

Evaluation of in-house PEG Precipitation Assay – Abbott Architect

ANALYTICAL EVALUATION OF MACROPROLACTIN ON ABBOTT ARCHITECT *i2000 SR* and *i2000* - Moses, Vandenberghe, Waite, and Sumner.

Method: Randomly selected male and female samples (n=100) with prolactin above the reference range (18-224 ug/L) were analyzed for prolactin following 1:2 dilution with Architect Multi-Assay Diluent and with 250 g/L PEG 6000 in same Architect diluent. Two methods of sample preparation were compared: A) precipitation in conical centrifugation tubes, ultracentrifugation at 3,750g and transfer of supernatant to cups with B) preparation in small polypropylene tubes (10x70 mm), centrifugation at 4,140g and direct sampling of supernatant by the Architect.

Results: Prolactin in PEG reagent was < 0.6ug/L. Correlation between straight and diluted prolactin was $Y=1.011X - 2.1$ ($r=0.997$). Following PEG precipitation the % recovery of prolactin was: * 55 patients had >60% prolactin recovery suggesting monomeric form, * 28 patients had <40% recovery consistent with macroprolactin and * 17 patients had between 40-60% recovery representing a mixture. Correlation between the 2 methods was $Y=1.071X - 0.90$ ($r=0.988$) and interpretation of prolactin recovery after PEG precipitation the same.

Conclusion: Prolactin and macroprolactin measurements are unaffected by dilution and PEG precipitation. Both sample precipitation techniques are acceptable for routine use in the primary care setting. The relative frequency of macroprolactin in our randomly selected group is consistent with those previously reported.

Performance Comparison of the Roche E170 PEG and Non-PEG Assays vs In-house PEP Architect Assay

- With Respect to MacroPRL (in spite of slightly different RI), we obtained 100% agreement between Beckman Dxi and the Architect PEG protocol for actual patients (n=25) with PRL > Architect URL (1 pt was in the gray zone by Architect & no MacroPRL present for the Dxi)
- For monmeric (total Prl), all elevated by Architect (n=28) were also elevated by Roche original method but only 23 were elevated by the new Roche mehtod (others were elevated for males but normal for females)
- Of the 28 elevated patients, MacroPRL was detected by the original Roche method (PEG) in 5. (19 %; consistent with previously reported values of 19 – 22)
- With use of appropriate RI, the new Roche appears to meet the manufacturer's claim of no interference from MacroPRL

Issues: MacroPRL – Cut-points

■ All PRL's > ULR

	Abbott	Beckman	Roche
– Males:	< 18	< 18	2 – 20
– Females	< 26	< 25	3 – 30
– F (Preg)	10 – 300	10 – 300	10 – 350

■ Macro PRL

- > 60 %, MPRL NOT Present (Supernatant)
- 40 – 60, % Mixture of Macro, Oligo & N-PRL
- < 40 %, MPRL Present (Supernatant)
- Opposite if the MPRL is measured on PPT (e.g. Beckman DX1 assay)

Endogenous/Exogenous Interferents – Digoxin: The Issues...

- What should Labs do if method is susceptible
 - Change method (not always an option)
 - Pre-treat samples to remove/minimize effects (not an options for automated methods)
 - Wait for Supplier to correct the problem & add an interpretive comment to all patient reports (option taken by most labs)
- Typical Comment (e.g.GDML's)

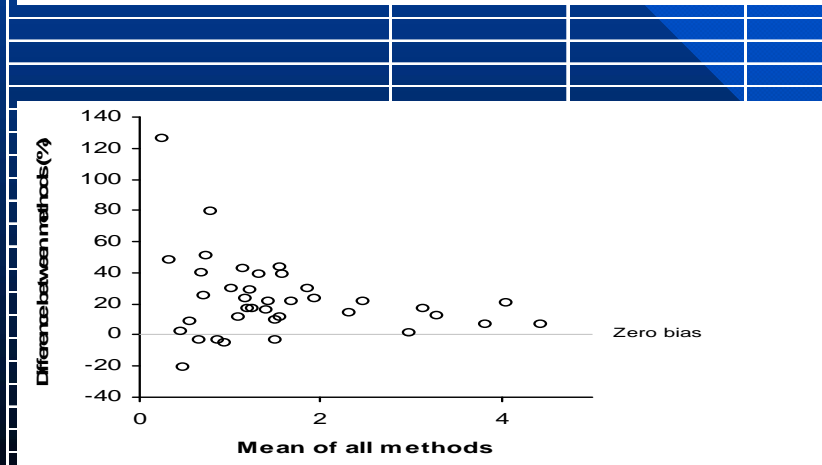
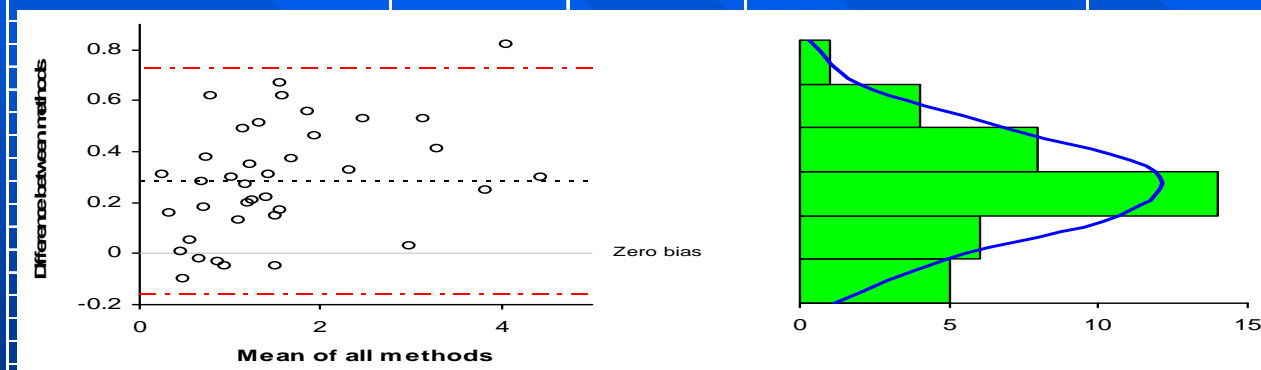
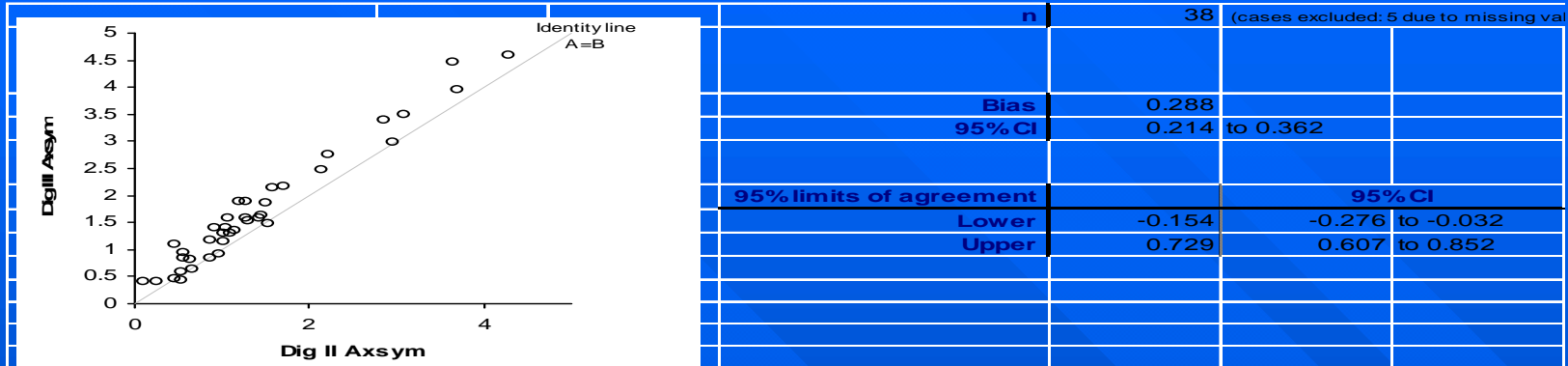
“Very recent authoritative studies show that digoxin results by most commonly used methods including ours, can be used suppressed up to 40% by spironolactone, canrenone, hydrocortisone and prednisolone. We will inform you when we change to a method not affected by these drugs”

Question – How do participants approach this issue in their Labs?

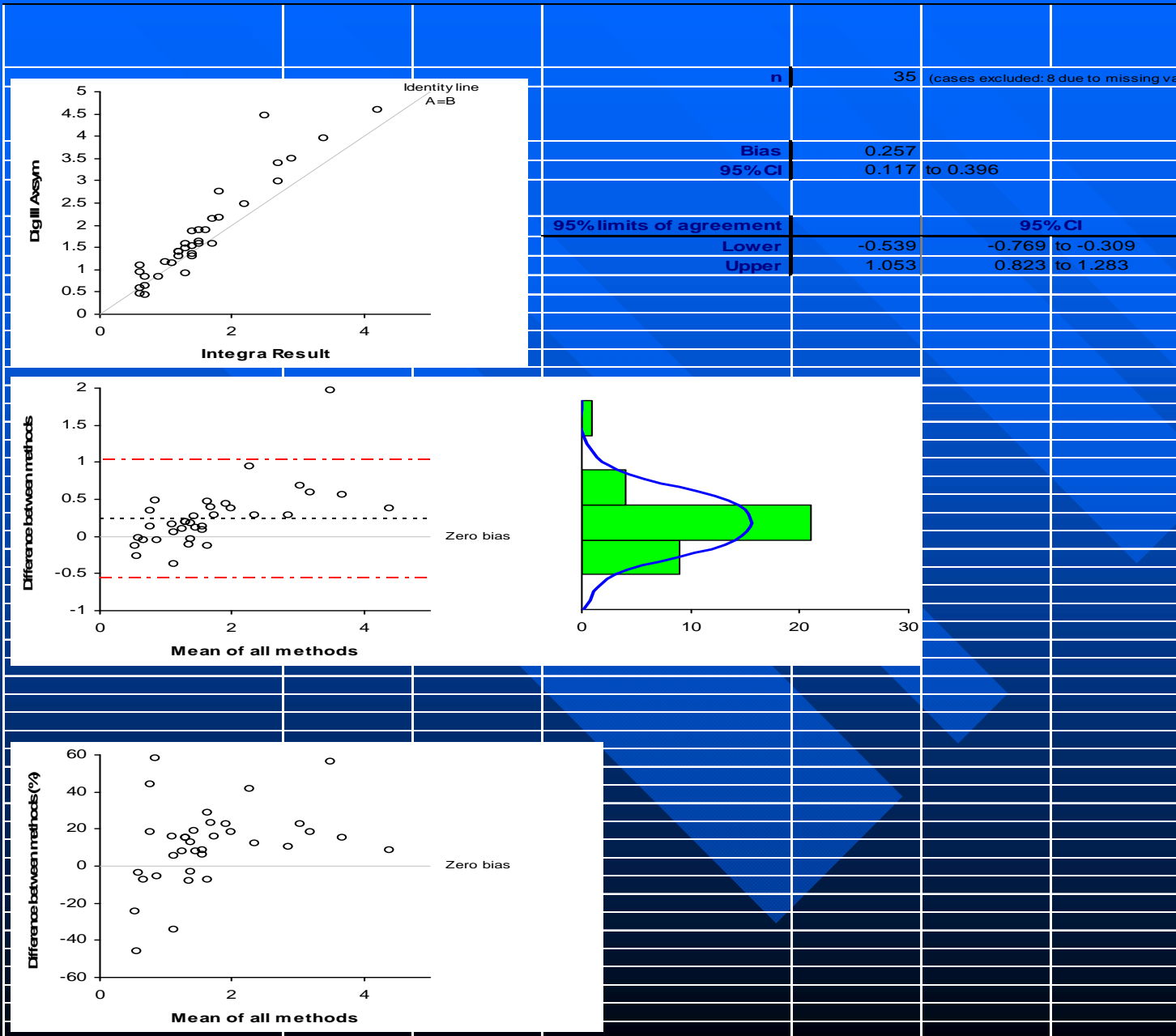
Endogenous/Exogenous Interferents – Digoxin; The Issues...

- **Waiting for Supplier creates other issues**
 - New formulation yields clinically different results from old formulation
 - Over/Under correction of the initial problem or reformulation introduced added error
 - Abbott AxSYM Dig III yield 30% higher results than Dig II
 - Also Claims that drug effect is no longer an issue with Dig III
- **Abbott Dig III results did not correlate with the Roche TIA or DPC Assays, which have been shown not to be affected by the drug**

Comparison studies – AxSYM III vs II



Comparison studies – AxSYM III vs Integra



Others - Antisera Specificity & Source/Type, ETC

- False Positives in Serological Assays
 - IgM due to RF & ANA Results
 - Fc Fragments of IgG Antibodies used with Enzyme conjugates (way around this is to use Fab fragments)
 - Anti-Toxoplasma IgG & IgM
 - » Major problem is IgM specific test lacks specificity
 - » A Specific IgG test is done in conjunction with IgM
 - » Results are interpreted in a binary manner
 - Anti-Rubella Antibody
 - » Similar problems but Immunoassays have improved specificity

Others - Antisera Specificity & Source/Type, ETC

- HAMA (Human anti-Mouse Antibody)
- HA (Heterophilic Antibodies)
- Manufacturer's disclaimers (Motherhood statements)
- Limitations to the procedures
- Cross-reactivity information
 - If negative effect, how does cross-reactivity determined?
 - Not the same as Interference testing
 - Not often quantifiable.

Conclusions

- PY Wong classified such issues as...
 - Exogenous (carryover, contaminants affecting label detection, additives in blood collection tubes & micro-clots)
 - Endogenous (Anti-analyte Antibodies, Human anti-mouse antibodies, Heterophilic antibodies, cross-reacting substance, high dose hook effect & non-specific interference)
 - All, or most of them, we encounter on an on-going basis in our day-to-day activities in the clinical laboratory
 - The presentation is a general overview of current issues in this wide & varied field. Hopefully, you will find the information useful.
 - I note with much amazement, the last major breakthrough (milestone in the development) in Immunoassays was Kohler & Milestein's monoclonal antibodies 1975 (32 years ago)!!!

Acknowledgements

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