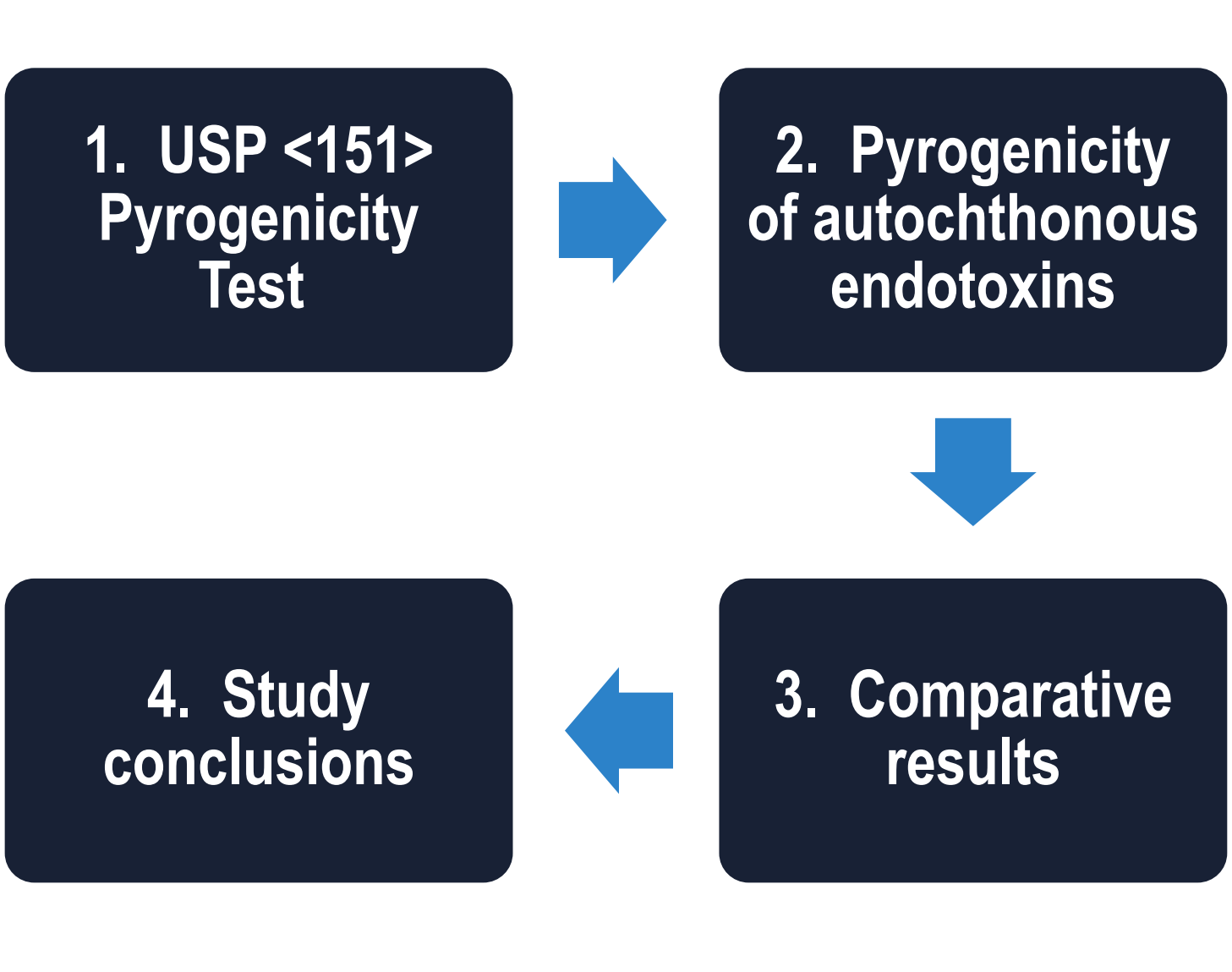
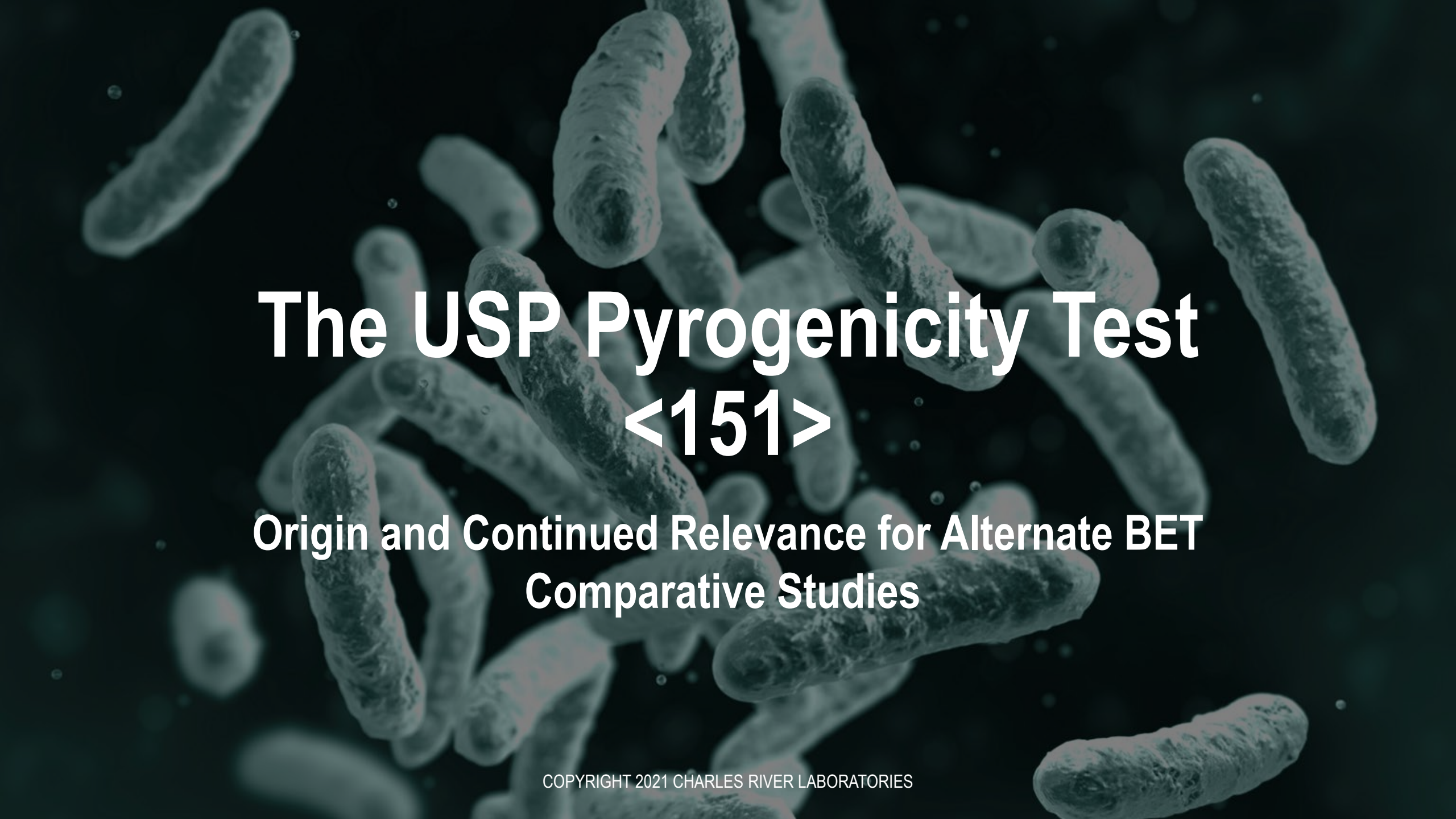
THE IMPORTANCE OF DATA COMPARABILITY OF LAL AND RECOMBINANT BET METHODS WITH NATURALLY CONTAMINATED PRODUCTS

Nicola Reid and John Dubczak

AGENDA





The USP Pyrogenicity Test <151>

Origin and Continued Relevance for Alternate BET Comparative Studies

PERSPECTIVE ON THE USP PYROGEN TESTING

- In 1941, the Committee of Revision of the USP authorized Sub-Committee 3 on Biological Assays carried out the first USP Collaborative Study of Pyrogens under the direction of Henry Welch:
  - Filtrates of *Pseudomonas aeruginosa* (from the FDA Division of Bacteriology)
  - FDA, NIH, and 14 pharmaceutical companies were involved
- The study involved:
  - 3,300 rabbit tests
  - 1782 tests with pyrogenic materials
  - 1017 with non-pyrogenic materials
- Results were published in 1943 and incorporated in USP XII
- Very little has changed in the rabbit testing protocol

CORRELATION OF THRESHOLD PYROGENIC DOSES

- Definitive work correlating the rabbit fever response to human fevers was published in 1969 by Greisman and Hornick:
- They compared 3 purified endotoxin preparations in rabbits and healthy human volunteers:

Endotoxin	Man (ng/kg)	Rabbit (ng/kg)
Pseudomonas species	50-70	50-70
E. coli	1.0	1.0
Salmonella typhosa	1 - 4	0.1- 0.14

GREISMAN & HORNICK FINDINGS

- Fever induction at **threshold** pyrogenic doses **were virtually equivalent** to humans and rabbits
- However, the endotoxin dose response relationship for humans is considerably steeper than those for rabbits
    - **Humans respond more vigorously to higher doses of endotoxins**
- Subjective toxin responses (chills) in humans increase sharply as endotoxin dosage are increased
- Authors also noted underlying illnesses that enhance the human pyrogenic and subjective toxin responses to endotoxin

SAFETY MARGIN IS APPLIED TO USP RABBIT TEST

"….Most users of the rabbit test employ the **threshold pyrogenic dose in rabbits as a minimum standard** for correlation with humans on a dose per weight basis and **attempt to increase the test safety margins for humans several times, if at all possible**".

Marlys Weary, *Pyrogens Endotoxin, LAL Testing, Depyrogenation;* Frederick C. Pearson

LAL's PROVEN SENSITIVITY AND SPECIFICITY

1942

Rabbit Pyrogen test enters USP

1971

Dr. James Cooper applies LAL Test to radiopharmaceuticals. Demonstrated That LAL Was 10 Times More Sensitive To Endotoxins Than The Rabbit Test

1973

FDA provides provisional use of the LAL test and allows industry to obtain real world experience with LAL.

1979[1] & 1982[2]

Baxter Healthcare provides annual testing totals as evidence of LAL superiority:

356,548 LAL test | 66,594 USP Rabbit Tests

404 Samples were contaminated with natural environmental endotoxins:

LAL confirmed all 404 failures | Rabbit Tests produced 19 / 404 failures

1983

USP replaces RPT with Bacterial Endotoxin Test

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ARE RECOMBINANT ALTERNATIVES EQUIVALENT TO LAL?

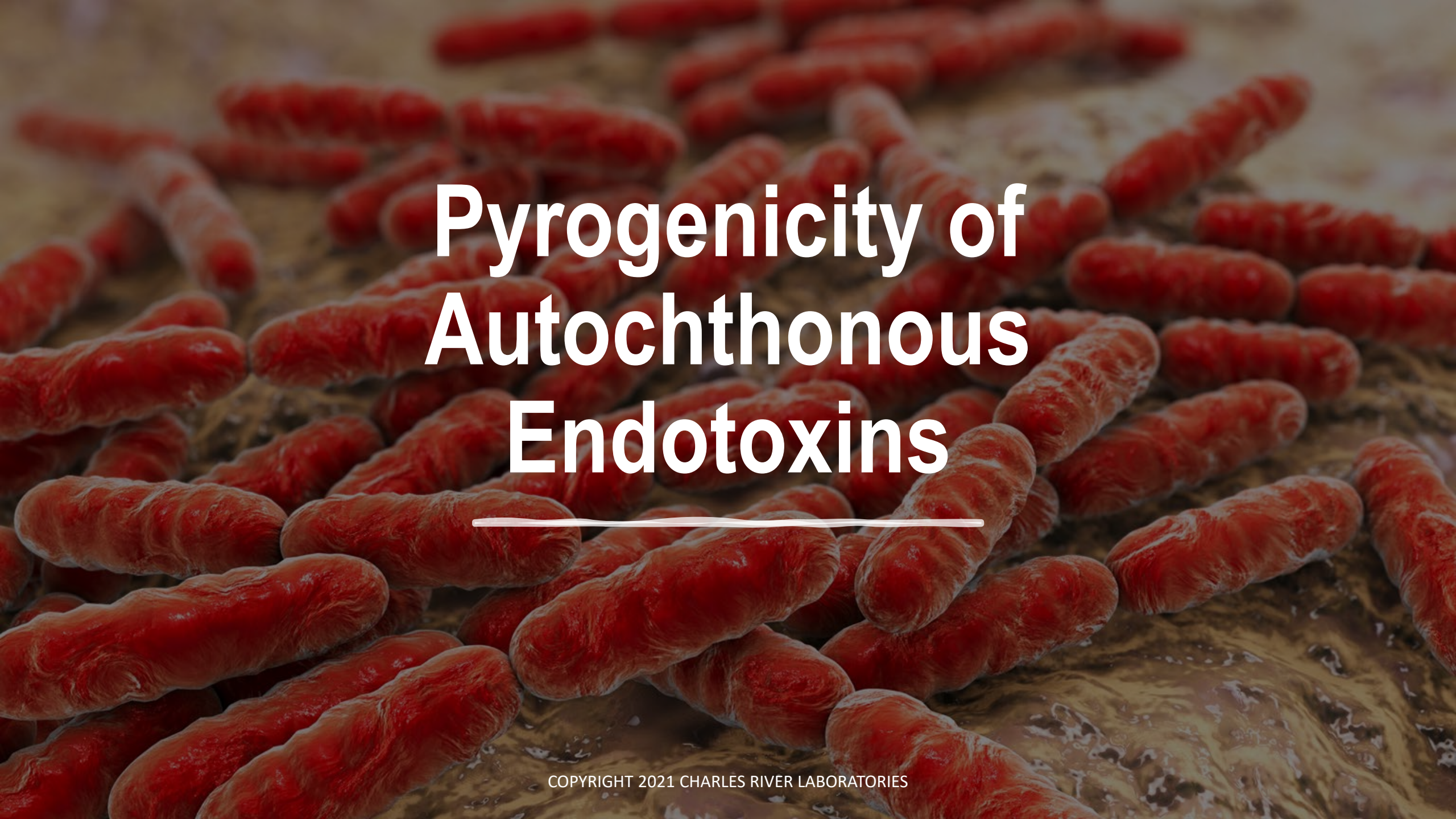
- Statements should reveal equivalent or higher endotoxin measures from samples contaminated with autochthonous endotoxins.
- Recombinant reagents capacity to underestimate autochthonous endotoxins concentrations have been noted:
  - Kikuchi et al[3]
  - Dubczak et al [4]
  - ACC Technical Report [5]

RATIONALE FOR USING THE USP RABBITS TEST

Rabbits provide direct evidence of a sample's capacity to induce an inflammatory response

Rabbits can serve as a "referee" for disparate LAL and recombinant endotoxin measures

Comparative testing that assesses the pyrogenicity of autochthonous endotoxin remains the most critical aspect for alternate BETs with respect to patient safety.



Pyrogenicity of Autochthonous Endotoxins

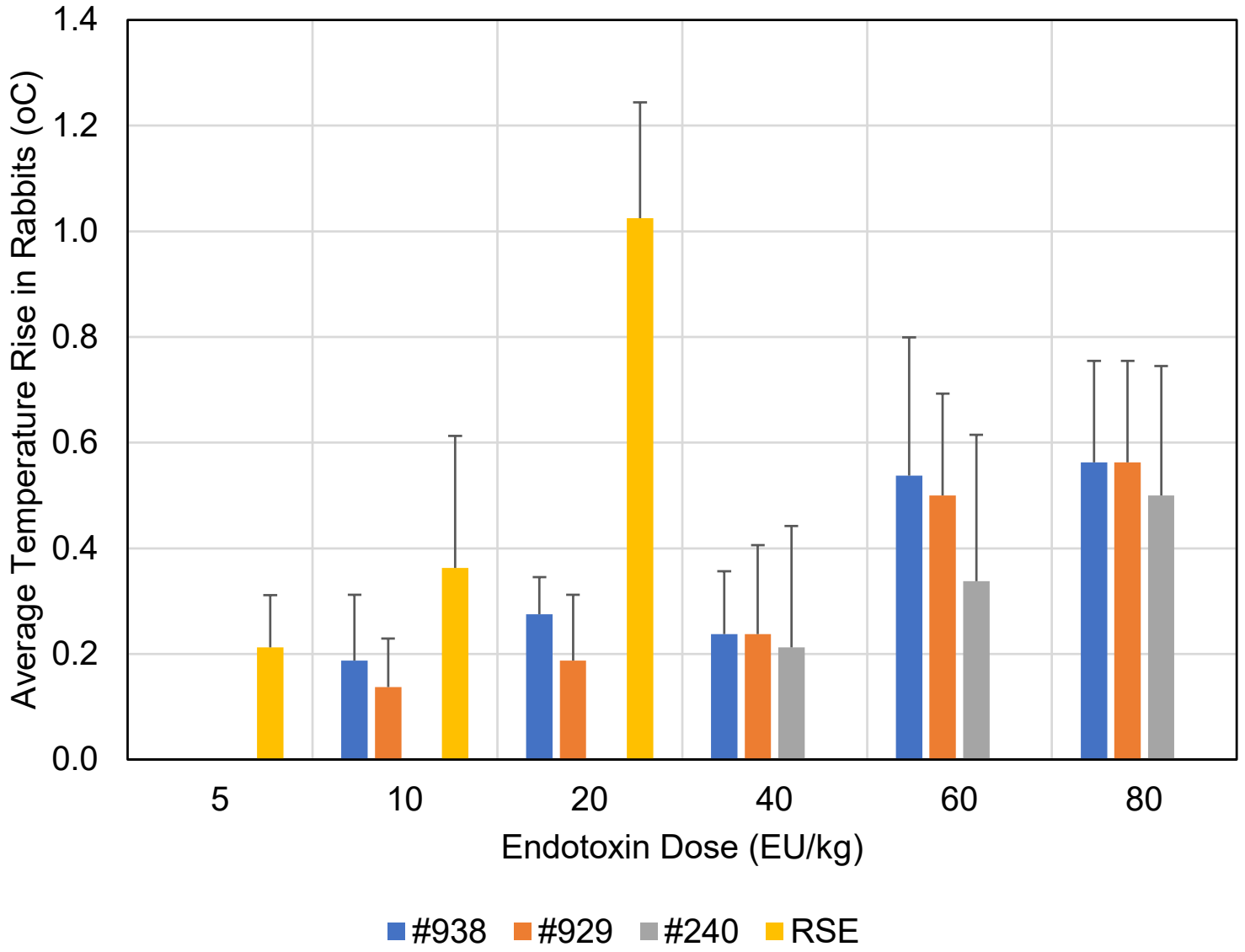
COMPARATIVE PYROGENICITY STUDY

- Established dose ranges for RSE and 3 samples containing autochthonous endotoxins:
  - Two-fold and/or four-fold dose ranging concentrations of each test article were used
  - 10 ml/kg test doses were administered for all test articles

PYROGENIC DOSE RANGING

Key Takeaways:

Activity of RSE is significantly higher in rabbits than autochthonous endotoxin.



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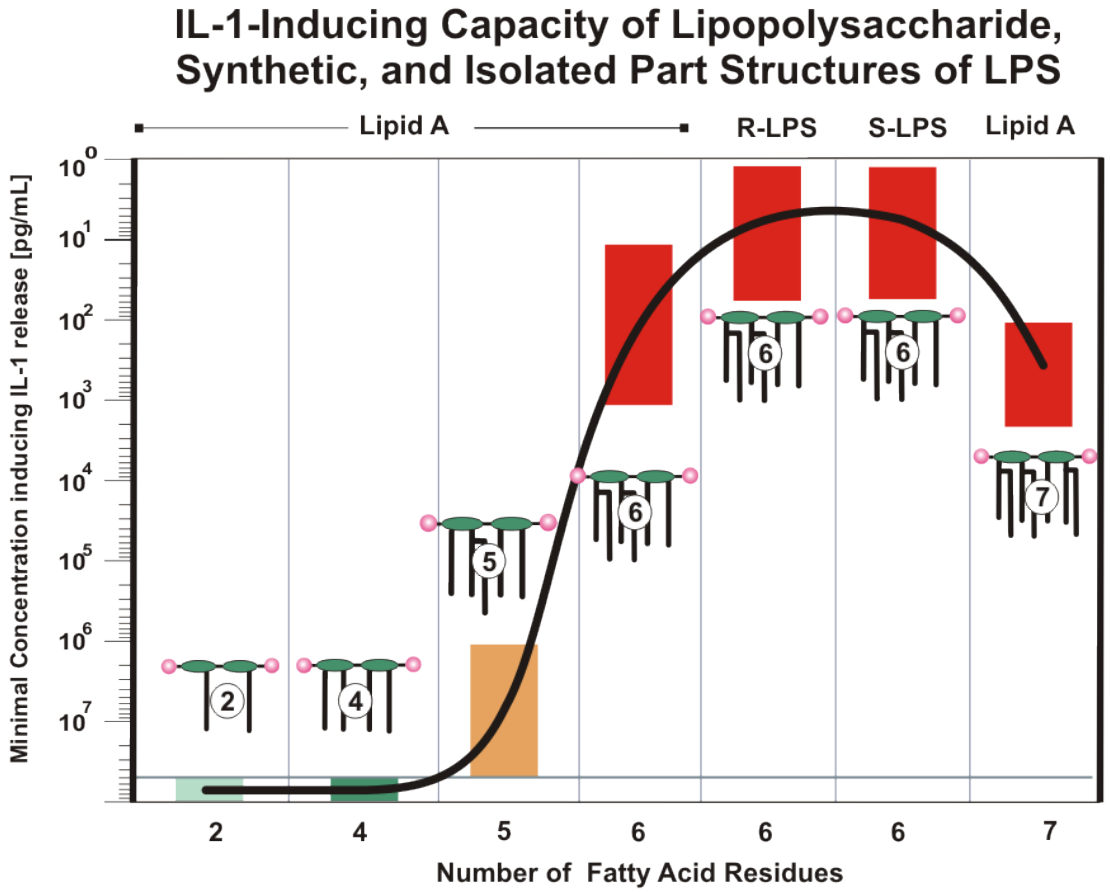
CONCLUSIONS FROM THE DOSE RANGING PYROGEN STUDIES

- The average pyrogenic response for RSE was consistent with the study conducted by Dr. Hochstein (FDA) for EC-2 in 1983
- The pyrogenicity of autochthonous endotoxins in water pre-treatment samples is different than RSE.
    - These data are consistent to observations made 30 years ago and consistent with we know to be critical for IL-1 induction

PYROGENIC ACTIVITY OF ENDOTOXINS

- Induction of IL-1 is highly dependent on LPS architecture:
  - Number of fatty acids
  - Negative electrostatic charge associated with the C1 and C4' phosphate moieties



H. Loppnow et al., *J. Immunol.* 1989,**142**,3229-3238

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LAL SENSITIVITY AND REACTIVITY

- LAL has been shown to be 2–75 times **more sensitive** to LPS than the rabbit pyrogen tests.
- Activation by LAL is not architecturally dependent.
- Rather, Factor C activation is largely determined by a localized conformational change when bound to the surface of Gram-negative bacteria and secreted LPS structures.

SAFETY FACTORS OF LAL TO USP RABBIT TESTS

Weary et. al 1982 Alan R Liss

Endotoxin	LAL Endpoint (pg/ml)	APD50 (pg/ml,10 ml/kg)	Sensitivity Ratio (USP<151>/LAL)
E. coli 0113 EC-2	46	94	2.0
E. coli 0113 EC-X	15	82	5.5
E. coli 055:B5	23	121	5.3
S. dysenteriae WHO	137	329	2.4
S. abortus equi (Novo Pyrexal)	9	57	6.3
A. calcoaceticus CDC	86	252	2.9
P. aeruginosa CDC	469	12,300	26.2
P. aeruginosa LIST	8	599	74.8
S. marcescens LIST	7	287	41
Y. enterocolitica LIST	27	61	2.3
V. cholerae LIST	29	1,729	59.6

Comparative Tests

COMPARATIVE RABBIT STUDY

- Four reagents simultaneously examined test articles with the USP 8 rabbit test:
  - One FDA Licensed LAL
  - Two rLAL formulations (Charles River)
  - One Recombinant Factor C Reagent
- Preparation of Test Articles:
  - Three samples with the target dose at 7 EU/ml (#240, 929, 938)
  - Two samples with the target dose at 4 EU/ml (#600, 650)
  - One sample with the target dose: <1 EU/ml (706)
  - Three carbohydrates samples prepared at a concentration of 5% (w/v)
- All test articles were diluted in Normal Saline and administered at 10ml/kg
- A single RSE curve was used for all reagents
- All assays were conducted at or near the same time

RESULTS: EU/mL MEASUREMENTS

Key Takeaways:

Highlighted in **Red** are samples showing underprediction compared to LAL

Highlighted in **Purple** are samples showing overprediction compared to LAL.

Highlighted in Black are values within the tolerance range of the LAL value.

Sample	Endotoxin detected in EU/mL (relative recovery compared to LAL)			
	KCA M3632E	rLAL (Form 1)	rLAL (Form 2)	rFC
#240	5.05 (100%)	**14.5 (287%)**	**10.44 (207%)**	**2.25 (45%)**
#929	4.55 (100%)	4.01(88%)	4.47(98%)	**0.81 (18%)**
#938	4.78 (100%)	9.43 (197%)	8.06 (169%)	**1.86 (39%)**
#600	3.25 (100%)	5.6(172%)	6.19 (190%)	**1.29 (40%)**
#650	4.26 (100%)	7.32 (172%)	8.21 (193%)	2.89 (68%)
#706	0.29 (100%)	0.54 (186%)	0.489 (168%)	**0.107 (37%)**
5% Sucrose (F)	0.55 (100%)	0.670 (122%)	0.703 (128%)	**0.149 (27%)**
5% Sucrose (K)	0.11 (100%)	0.209 (196%)	**0.260 (243%)**	**0.048 (45%)**
5% Lactose (K)	0.16 (100%)	0.159 (100%)	0.245 (154%)	**0.019 (12%)**

Note: One sample is under investigation

EU/ml MEASUREMENTS - RECOMBINANT REAGENTS ONLY

GLUCAN BIAS IS NONEXISTENT

DISPARATE VALUES SEEN BETWEEN RECOMBINANT REAGENTS

Sample	rLAL (1)	rLAL (2)	rFC
#240	14.5	10.44	2.25
#929	4.01	4.47	0.81
#938	9.43	8.06	1.86
#600	5.6	6.19	1.29
#650	7.32	8.21	2.89
#706	0.54	0.489	0.107
5% Sucrose (F)	0.670	0.703	0.149
5% Sucrose (K)	0.209	0.260	0.048
5% Lactose (K)	0.159	0.245	0.019

EU/mL RESULTS OF RABBIT POSITIVE SAMPLES

Key Takeaways:

LAL and rLAL provides sensitivity, hence why no LAL false negative failures have occurred in the lifetime of LAL.

rFC demonstrates a lower sensitivity due to underestimation of autochthonous endotoxin compared to LAL.

Threshold
Pyrogenic Dose = 5EU/kg
Dosed at 10mL/kg gives 0.5EU/mL limit here.

Sample	KCA Lot M3632E (EU/mL)	rLAL (1) (EU/mL)	rLAL (2) (EU/mL)	RFC (EU/mL)	Sum of 8 Rabbits Failures (10 ml/kg) (°C)
#240 (Target 70EU/kg)	5.05	14.50	10.44	2.25	5.1
#929 (Target 70EU/kg)	4.55	4.01	4.47	0.81	4.3
#938 (Target 70EU/kg)	4.78	9.43	8.06	1.86	4.6
#600 (Target 40EU/kg)	3.25	5.60	6.19	1.29	8.2
#650 (Target 40EU/kg)	4.26	7.32	8.21	2.89	9.6

4.55/0.5 = 9.1x

0.81/0.5 = 1.6x

Study Conclusions

CONCLUSIONS

- Autochthonous endotoxins have been shown to be less potent than RSE yet are readily detected by LAL.
- Underprediction of autochthonous endotoxins result in the reduction of sensitivity and presents a patient safety risk.
- It is not adequate to compare alternate BETs to LAL using only laboratory prepared standards which are irrelevant with respect to patient safety. They simply do not exist in the pharmaceutical manufacturing environment.
- Alternate tests to the compendial need to have a clear advantage if they can't demonstrate equivalency. Is reducing a sensitivity to autochthonous endotoxins that has protected the public for four decades acceptable?

Contact Us:

John Dubczak: John.Dubczak@crl.com
Nicola Reid: Nicola.Reid@crl.com

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