(Latest) Challenges in the field of endotoxin and pyrogen testing

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Endotoxin and Pyrogen Testing
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Outline

I. Introduction

II. Case studies

1. Detection of endotoxin from E.coli and P.aeruginosa
2. Effects of sample matrix on test system
3. LER Hold time study

III. Discussion

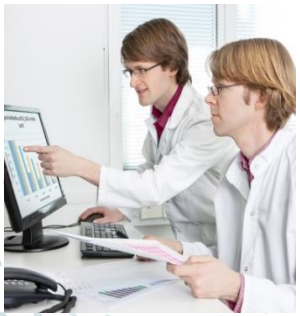
1. Heterogeneity of endotoxin and pyrogen tests
2. Heterogeneity of endotoxin

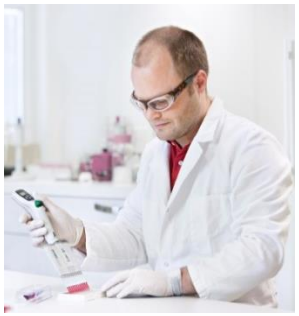
IV. Take Home Message

Introduction

Company overview

1992	Founded as a spin-off of Boehringer Mannheim GmbH; Main technology: coating of microwell plates
1995	Launch of protein chemistry branch: purification and modification of antigens & antibodies and conjugates
1996	Certification according to ISO 9001
1998	Launch of laboratory services: assay development, validation and analytical service for the diagnostic and pharmaceutical industry
2004	Certification according to ISO 13485
2009	Registration as GLP test facility
2013	Preferred provider for Biomarker studies for global leading Pharma company
2014	Establishment of **endotoxin testing** (protocol development)





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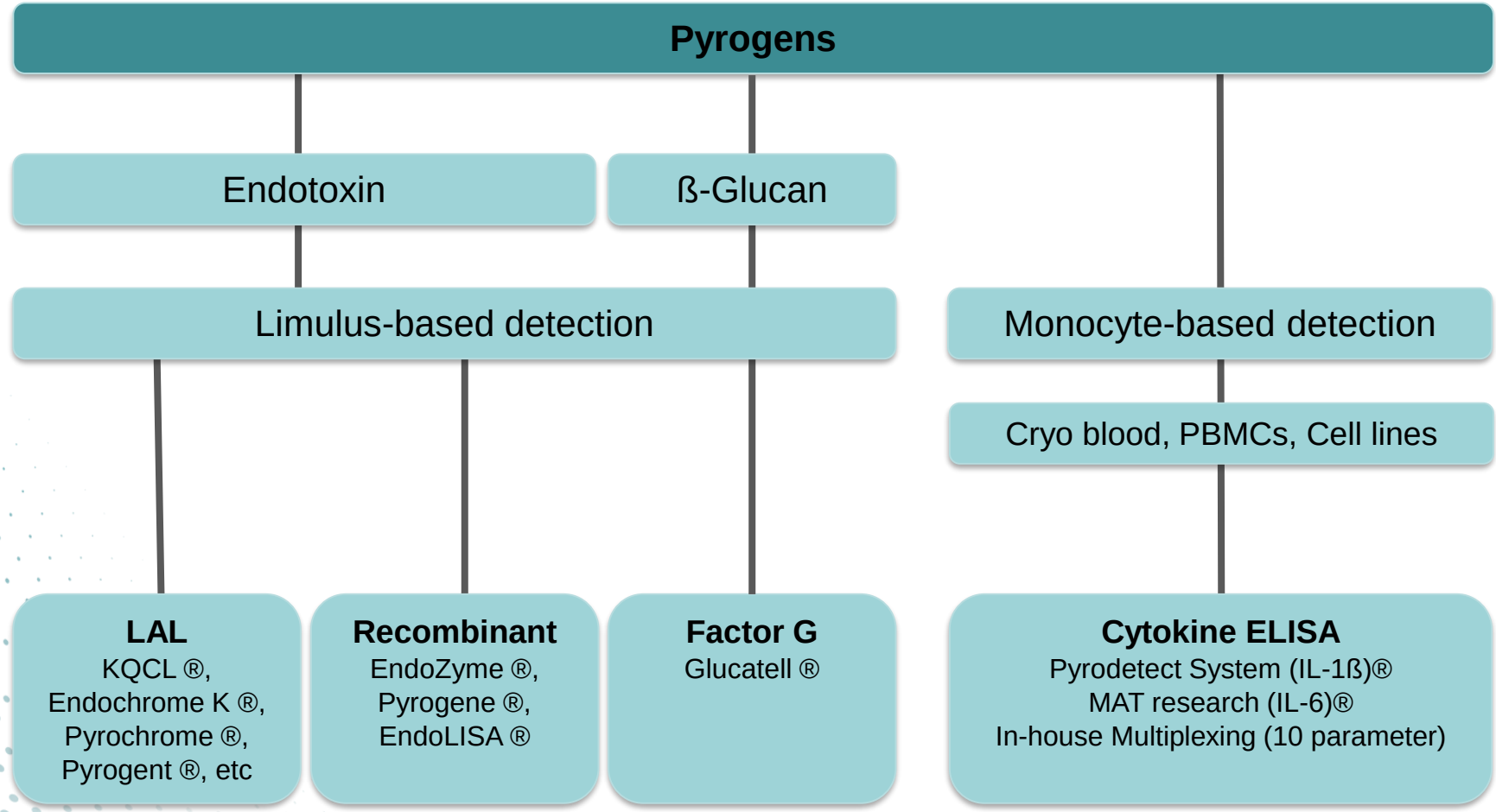
GLP

Introduction

Endotoxin and Pyrogen detection at Microcoat



Introduction

Definitions

Endotoxin	= Lipopolysaccharide (LPS)
RSE	= Reference Standard Endotoxin
LRW	= LAL Reagent Water
LER	= Low Endotoxin Recovery
MAT	= Monocyte Activation Test
Limulus-based tests	= Recombinant (Factor C) and LAL
Test method	= Limulus-based test and MAT

Case study 1

Detection of endotoxin from E.coli and P.aeruginosa

Conditions

Analysis of two samples:
- E.coli suspension
- P.aeruginosa suspension

Detection systems:
- LAL (KCA)
- rFC (EndoZyme)
- rFC (EndoLISA)
- MAT (IL-1ß)

Case study 1

Analysis of E. coli suspension

		LAL (KCA)		rFC (EndoZyme)		rFC (EndoLISA)			MAT (IL 1-β)	
Sample	**Dilution**	**Value [EU/mL]**	**PPC [%]**	**Value [EU/mL]**	**PPC [%]**	**Value [EU/mL]**	**PPC [%]**	**Dilution**	**Value [EU/mL]**	**PPC [%]**
1	5	4.14	136	5.73	120	8.75	125	1	1.28	-60
1	25	4.45	147	5.67	102	8.05	130	2	2.43	-66
1	100	4.32	157	5.10	64	8.60	128	20	4.96	131
1	500	3.27	129	5.00	87	<25	110	50	<62.5	107
mean		**4.05**		**5.38**		**8.47**			**4.96**	

→ **Comparable results are obtained, independent of test method.**

Case study 1

Analysis of P. aeruginosa suspension

		LAL (KCA)		rFC (EndoZyme)		rFC (EndoLISA)			MAT (IL 1-β)	
Sample	**Dilution**	**Value [EU/mL]**	**PPC [%]**	**Value [EU/mL]**	**PPC [%]**	**Value [EU/mL]**	**PPC [%]**	**Dilution**	**Value [EU/mL]**	**PPC [%]**
2	5	162	N/A	119	121	131	76	10	2.96	69
2	25	216	N/A	156	113	162	112	50	<6.25	82
2	100	305	38	160	83	157	144	100	<12.5	100
2	500	360	144	158	102	130	153	500	<62.5	92
mean		**360**		**148**		**150**			**2.96**	

→ **Limulus-based test show comparable results**

→ **MAT measures substantial lower activity than Limulus-based tests**

Case study 2

Effects of sample matrix on the test system

Conditions:

Analysis of two samples:
- 5 EU/mL RSE in LRW
  1:10 dilution
  0.5 EU/mL PPC (CSE)
- 5 EU/mL RSE in drug product
  1:50 dilution (MVD)
  0.5 EU/mL PPC (CSE)

Detection systems:
- LAL KCA (Vendor 1)
- LAL KCA (Vendor 2)
- LAL KCA (Vendor 3)
- LAL KTA (Vendor 1)

Case study 2

Analysis of RSE in LRW

Test	Sample 1 [EU/mL]	PPC [%]
LAL KCA (Vendor 1)	7.25	145
LAL KCA (Vendor 2)	3.75	105
LAL KCA (Vendor 3)	6.50	78
LAL KTA (Vendor 1)	3.70	137

→ **All tests show valid and comparable results** (within factor 2)

Case study 2

Analysis of RSE in a drug product

Test	Sample 2 [EU/mL]	PPC [%]
LAL KCA (Vendor 1)	1.69	99
LAL KCA (Vendor 2)	3.15	472
LAL KCA (Vendor 3)	2.9	216
LAL KTA (Vendor 1)	0.9	64

→ **Tests do not show comparable results**
→ **Different test systems behave different in a complex sample**

Case study 3

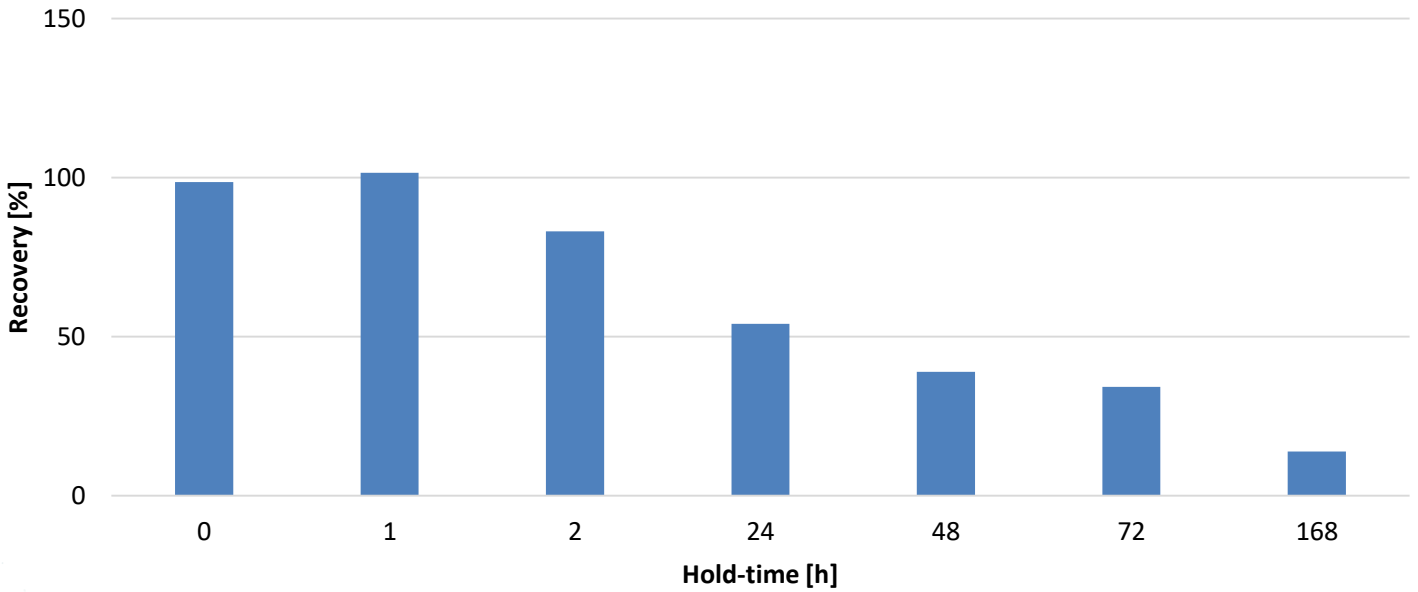
Low Endotoxin Recovery

Conditions:

LER Hold-time study:	Drug product In-process control Formulation buffer
Hold-time:	7 days
Temperature:	20°C – 25°C
Spike:	Reference Standard Endotoxin (RSE)
Spiking set-up:	Multi-aliquot + Reverse mode
Detection system:	LAL KCA

Case study 3

LER hold-time study on drug product



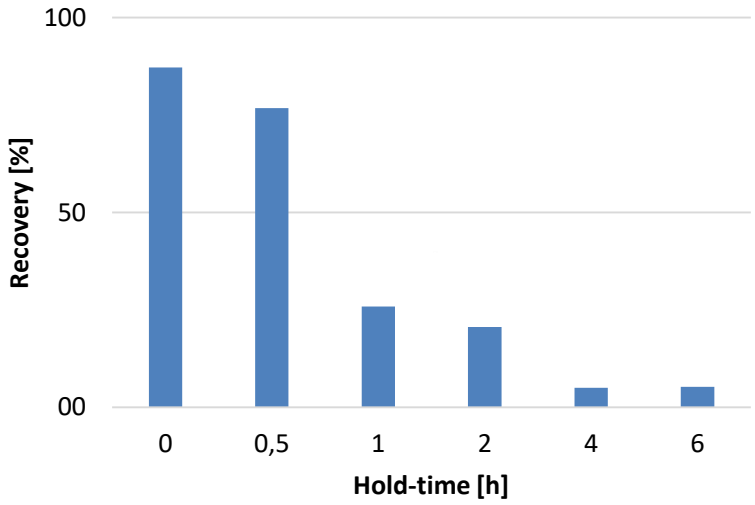
After 24 hours of LER hold-time, recovery < 50 %

→ **Drug product is affected by LER**

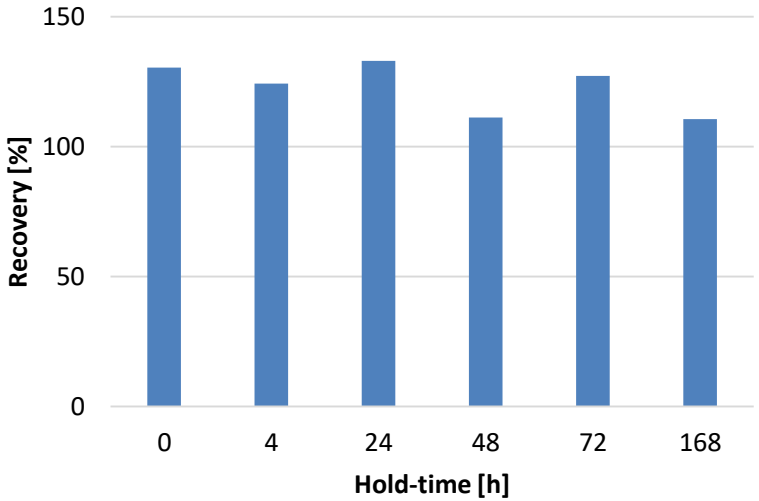
Case study 3

Investigation of LER driving forces

In-process control (IPC)



Formulation Buffer



Over 168 hours of LER hold-time, recovery **> 50 % in formulation buffer**

→ **Formulation buffer is not affected by LER**

After 0.5 hours of LER hold-time, recovery **< 50 % in IPC**

→ **IPC is affected by LER**

➔ **Endotoxin masked by protein**

Case study 3

Improvement of endotoxin detection

1) Higher sample dilution:	Recovery < 50%
2) Dilution with dispersing agent:	Recovery < 50%
3) Change of vendor for test system:	Recovery < 50%
4) EndoLISA + EndoRS (A+B+D+E)	Recovery **>** 50%
5) LAL KCA + EndoRS	Recovery **>** 50%

optimization of demasking approach by adjustment of component A (pH), B (destabilization of masking complex, E (reconfiguration of aggregates) and D (support reconfiguration of aggregations).

Different approaches tested to overcome LER

- ➔ **Endo-RS most effective approach**
- ➔ **Compatible with LAL, but further adjustments needed** (Case by case)

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Discussion - Heterogeneity of endotoxin and pyrogen test

Limulus-based detection vs. MAT

Different test methods show comparable detection of endotoxin in **E.coli** suspension

Limulus-based test systems show comparable detection of endotoxin in **P.aeruginosa** suspension

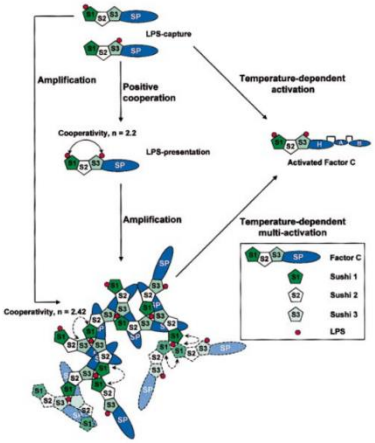
Different test methods do <u>not</u> show comparable detection of endotoxin in **P.aeruginosa** suspension (Limulus-based vs. MAT)

➔ Is there a difference between detection methods?

Discussion - Heterogeneity of endotoxin and pyrogen test

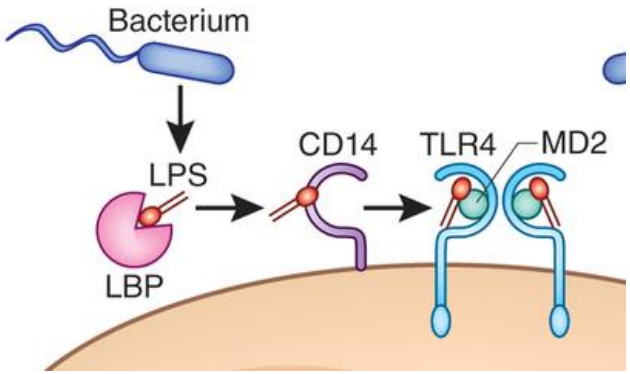
Reaction mechanisms of different detection methods

Factor C (Limulus-based tests):



Source: Tan et al. FASEB J, 14, 1801-1813, 2000

TRL-4: (MAT)



Source: Zipfel et al., Nat Immunol,16:340-341, 2015

Different detection methods underlie different reaction mechanism (Limulus-based vs MAT)
→ Varying reactivity may occur

In order to compare different methods:
➔ Test methods are calibrated against RSE
➔ RSE (Endotoxin from E.coli) serves as benchmark

Discussion - Heterogeneity of endotoxin and pyrogen test

Variations between Limulus-based test systems (Factor C)

Observations:

- Different Limulus-based test systems show comparable detection of RSE in H2O.
- In a complex sample matrixes, validity of results depend on test system and vendor
- In rare cases, changing LAL vendor/system solved the LER phenomenon
  (data not shown)

The common reaction mechanism in all Limulus-based detection systems is the activation of Factor C.

➔ **Why do we see in certain cases considerable differences between Limulus-based tests?**

Discussion - Heterogeneity of endotoxin and pyrogen test

The source of LAL (Limulus Amebocyte Lysate):

Picture taken at Pickering Beach, DE, USA, 2016

*Source: https://cdn.theatlantic.com**

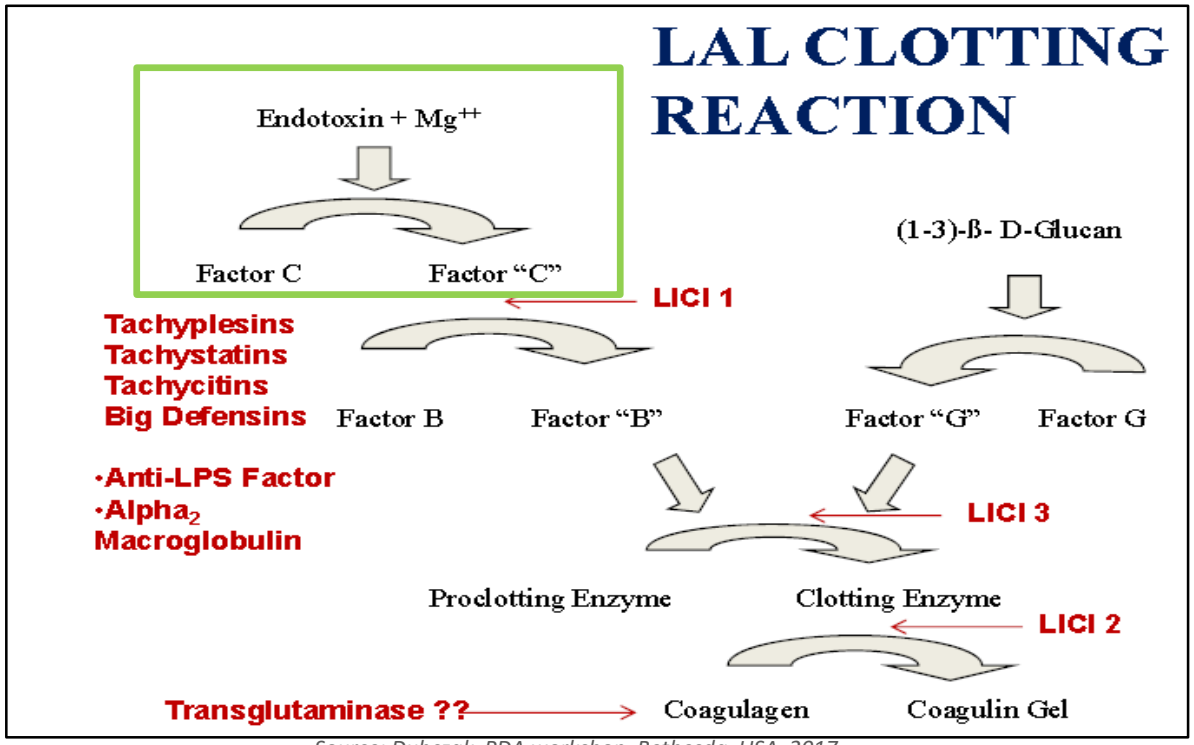
LAL tests are derived from blood of Horseshoe Crabs

→ **Variations due to the natural source are likely**

*https://www.theatlantic.com/technology/archive/2014/02/the-blood-harvest/284078

Discussion - Heterogeneity of endotoxin and pyrogen test

The LAL reaction



Source: Dubczak, PDA workshop, Bethesda, USA 2017

Lysate consists of complex mixture of components

➔ **The entire interplay of all components is not known**

Discussion - Heterogeneity of endotoxin and pyrogen test

Source and preparation of Limulus-based tests

LAL:

Horseshoe Crab

rFC:

Fermentation

Preparation + concentrations
+ formulation excipients

Assay reagent

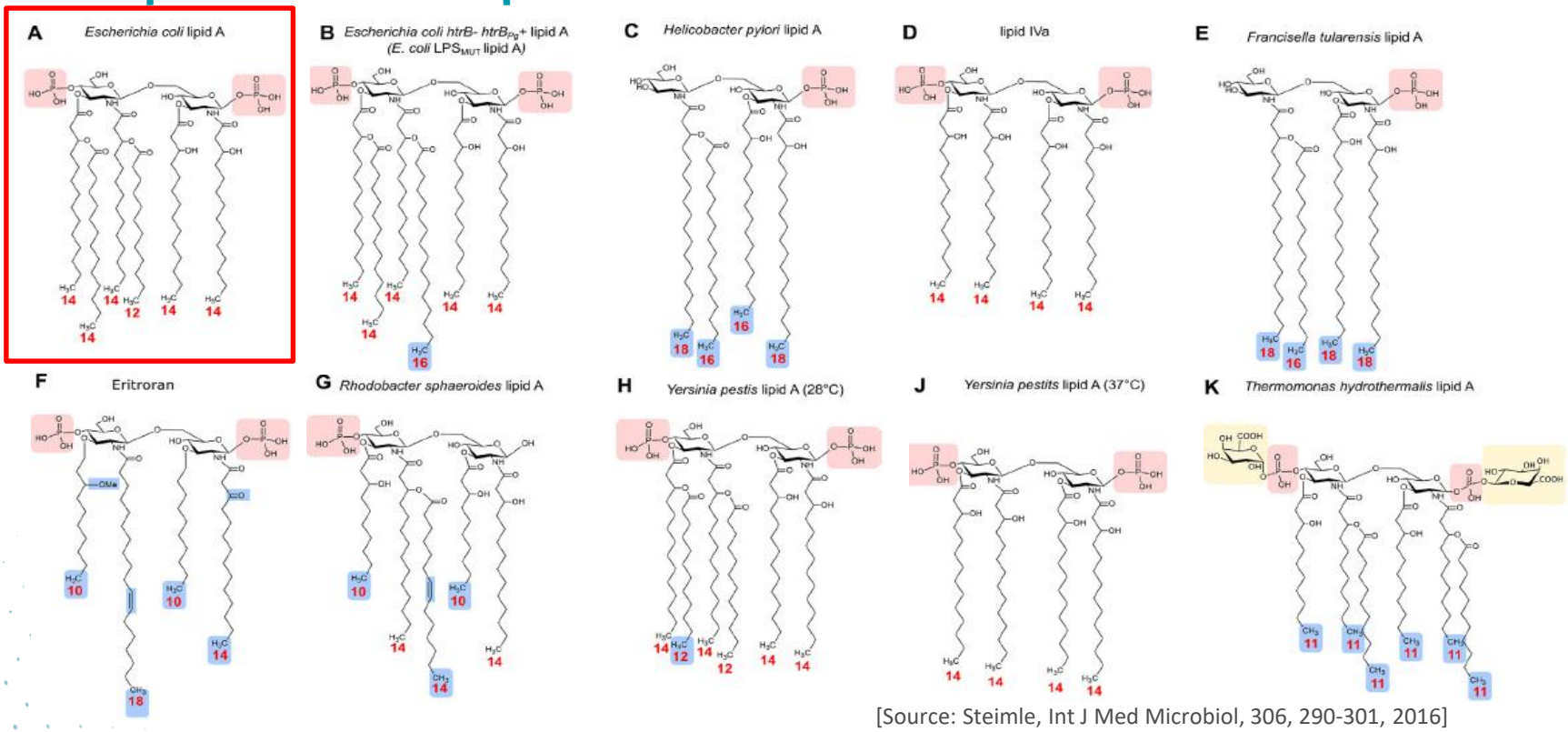
Preparations and formulations of assay reagents are proprietary information

For stabilization assays may be using formulated and include excipients like buffers, salinity, surfactants, etc.

➔ **Variations of assays might be due to individual preparations and formulations of assay reagents**

Discussion – Heterogeneity of endotoxin

Examples of different Lipid A structures of various bacteria



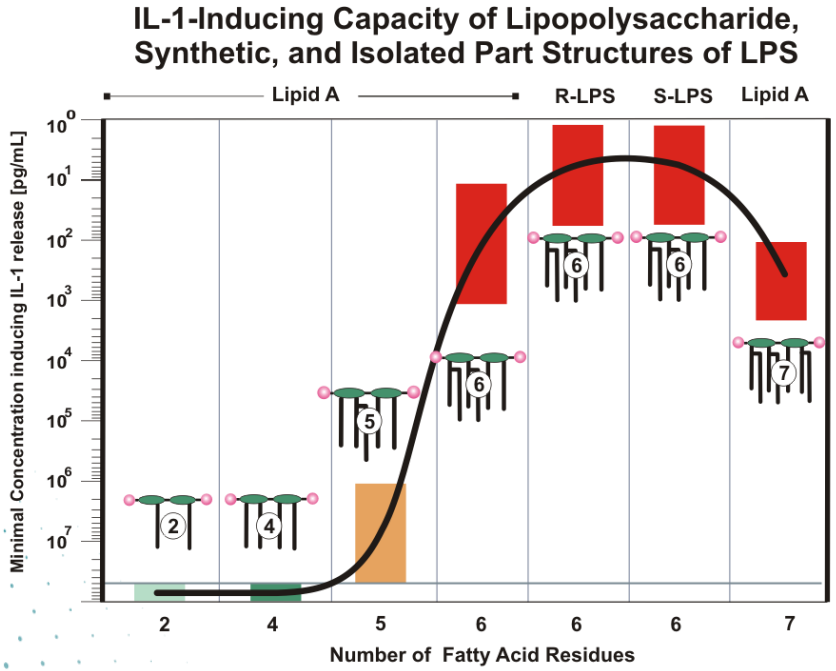
[Source: Steimle, Int J Med Microbiol, 306, 290-301, 2016]

Lipid A is the „toxic" part of LPS

→ **Pyrogenicity depends on structure**

Discussion – Heterogeneity of endotoxin

Molecular structure of LPS determines pyrogenicity



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

Greisman and Hornick showed that a threshold pyrogenic response level for E.coli is approx. ***50 times*** *higher than for Pseudomonas*
(In Proc. Soc. Exp. Biol. Med, 1969)

Hexa-acylated lipid A reflects most reactive LPS species

LAL-based methods may not show the same behavior as MAT (see Case study 1)

→ **Different reactivities in different detection methods** (eg. MAT vs. BET)

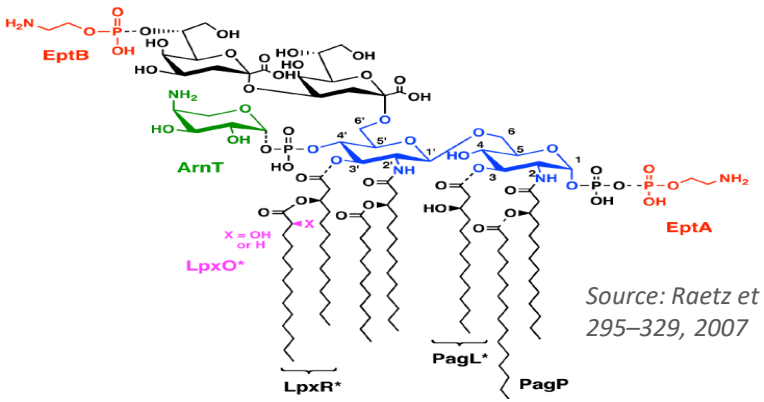
Discussion - Heterogeneity of endotoxin

Modifications of endotoxin

In addition to bacteria species-dependent molecular structures, modifications of LPS can occur during growth

For example:

- Change in phosphorylation
- Decoration with amino sugars, ethanolamine, amino acids
- Variation in amount, length, saturation of fatty acids



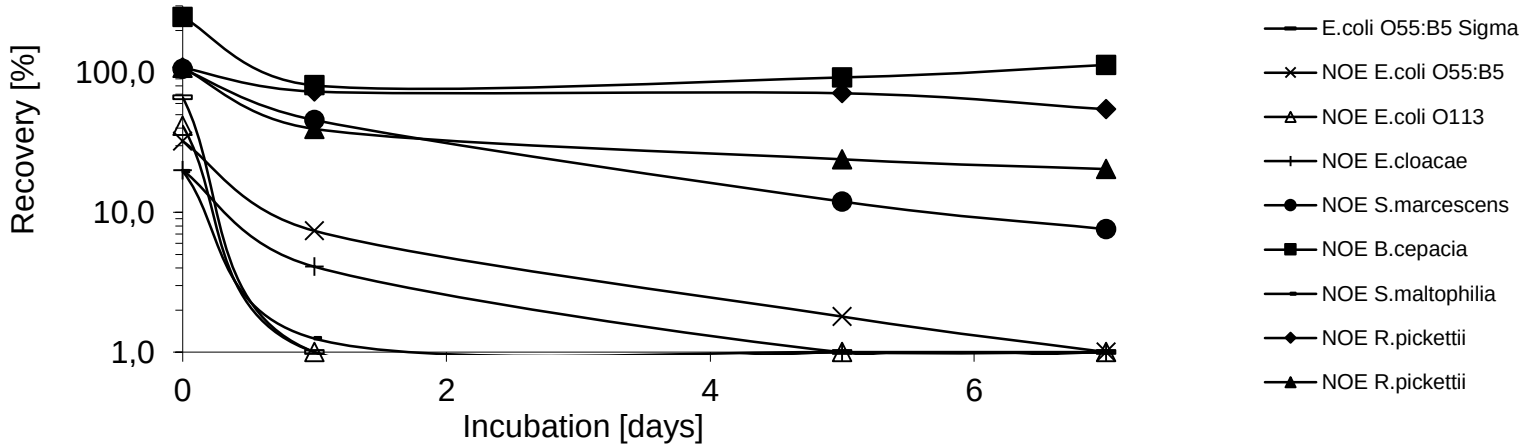
Source: Raetz et al., Annu Rev Biochem, 76: 295–329, 2007

➔ **One bacteria can contain up to 50 different sub-species of Lipid A**
(Trent, PDA workshop, Bethesda, 2017)

Discussion – Heterogeneity of endotoxin

Further effects due to heterogeneity of endotoxin

→ Different masking susceptibilities in case of LER



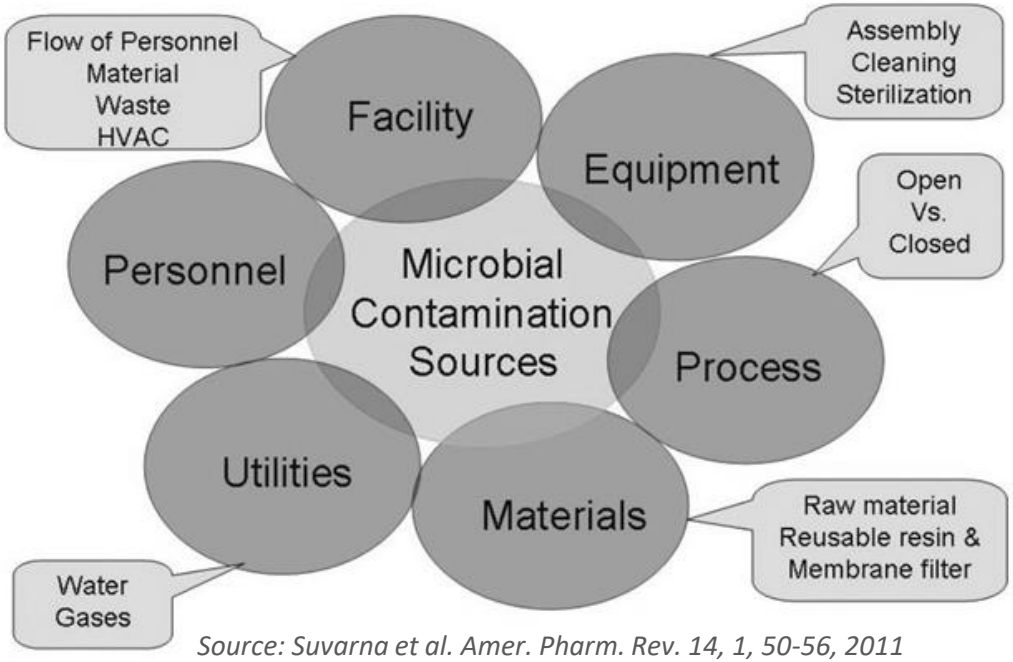
[source: Reich, ECA Webinar, 2015]

Endotoxins from different sources have been shown to behave different in LER hold-time studies

→ **Which endotoxin should be used for spiking?**

Discussion – Heterogeneity of endotoxin

Potential sources of microbial contaminations



Source: Suvarna et al. Amer. Pharm. Rev. 14, 1, 50-56, 2011

The source and structure of a bacterial/endotoxin contamination is unpredictable

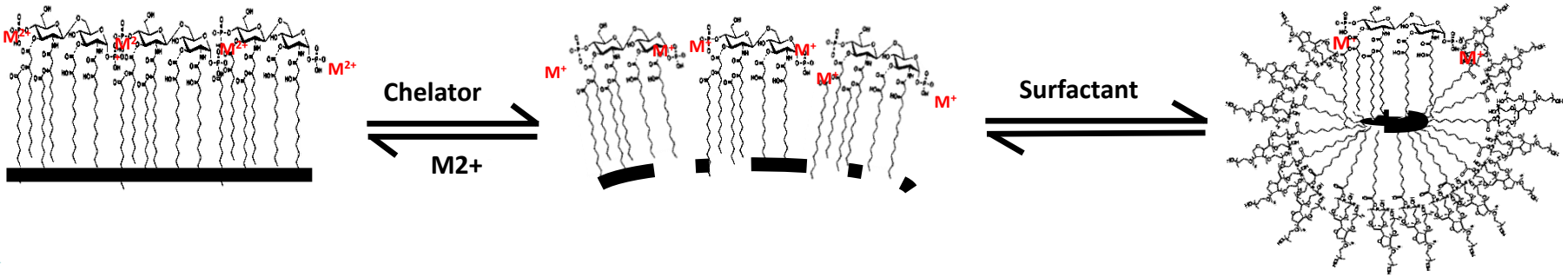
→ In order to identify the LER capability of a sample a susceptible endotoxin is needed

→ Endotoxin from E.coli (CSE/RSE) has been shown susceptible to LER

→ RSE recommended for LER hold-time studies

Discussion – Recap of day 1

Two-step reaction mechanism of LER



- Addition of M2+ prior to endotoxin spike prevents LER
- LER = time dependent phenomenon
- Chelation step is time-limiting

➔ **Supplementation of M2+ beneficial as long as the aggregation state is NOT altered by surfactants**

Take Home Message

- ❑ Endotoxin from different sources can substantially vary in its molecular structure

→ Depending on the molecular structure, endotoxin

- can react unequal in different test methods (e.g. LAL vs. MAT)
- possess different masking susceptibilities (LER)

→ Source and structure of potential contamination is not predictable

- ❑ RSE is benchmark for endotoxin and pyrogen testing

→ Calibration of test systems and methods

→ Spike for LER hold-time studies

Take Home Message

- Different test methods underlie different reaction mechanism
  - MAT → reaction pathway via toll-like receptors
  - Limulus-based tests → reaction pathway via Factor C
  - ➢ Analysis of the same sample may lead to different results

- Similar test systems, but from different vendors may lead to different results
  - Varying preparations and formulations may cause differences
    e.g. Salinity, Surfactants, …
  - ➢ For LER hold-time studies the same assays has to be used as for release testing

➔ **"Real" standardization of detection methods and systems desired**

Acknowledgement

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Julian Eifler

Lisa Eßbach

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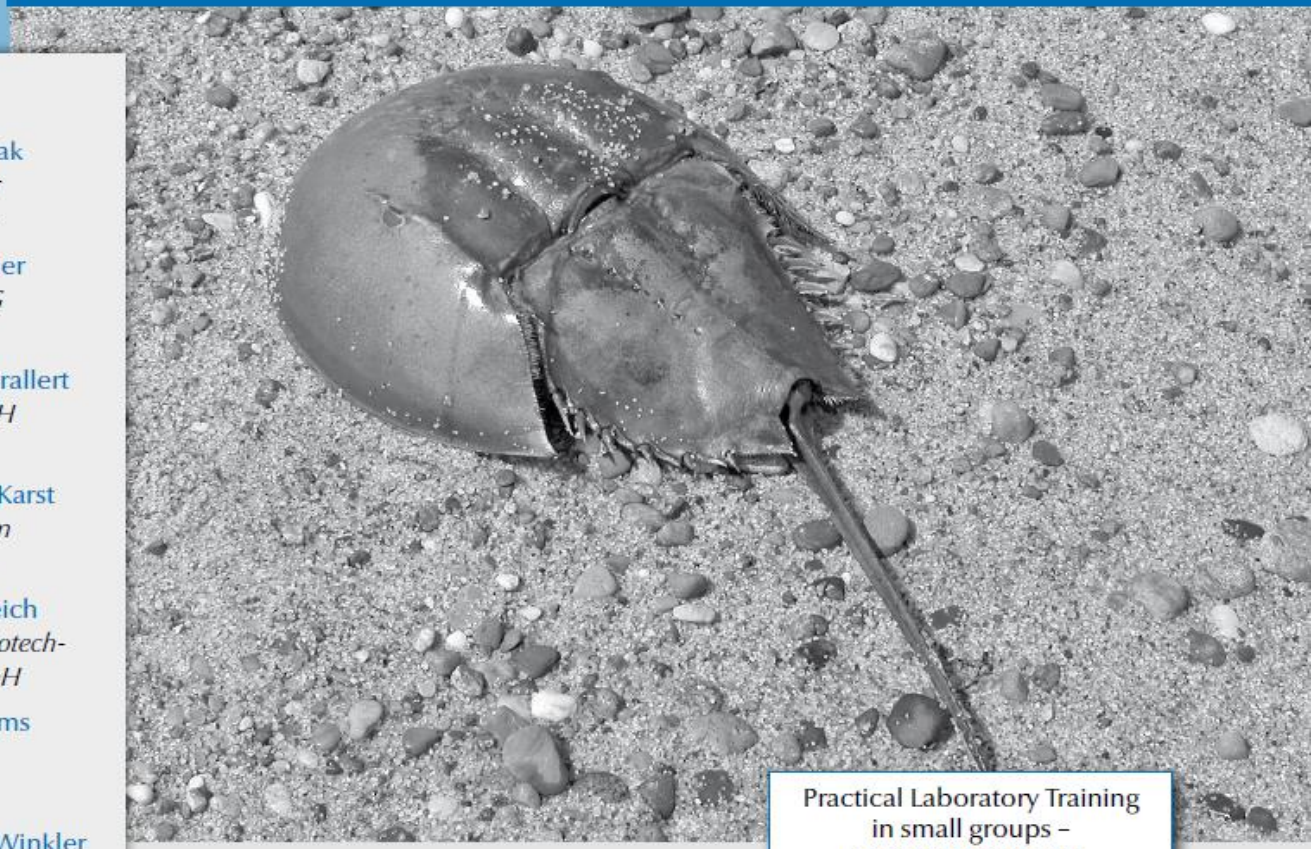
Low Endotoxin Recovery/Masking

Hands-on Laboratory Training Course



Practical Laboratory Training in small groups – max 15 participants.

27-28 February 2018, Munich/Bernried, Germany

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John Dubczak
Charles River Laboratories


Stefan Gärtner
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Dr Holger Grallert
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