Immunization of broiler chickens against necrotic enteritis: Progress and possibilities

John Prescott, Ravi Kulkarni

Necrotic enteritis

- Small intestinal infection of broiler chickens
- Caused by *Clostridium perfringens*
- Serious infection, through mortality and morbidity
- $6 billion dollar disease
- High cost of subclinical disease and of prevention by antibiotics
- Need to stop preventive use of medically-important antibiotics

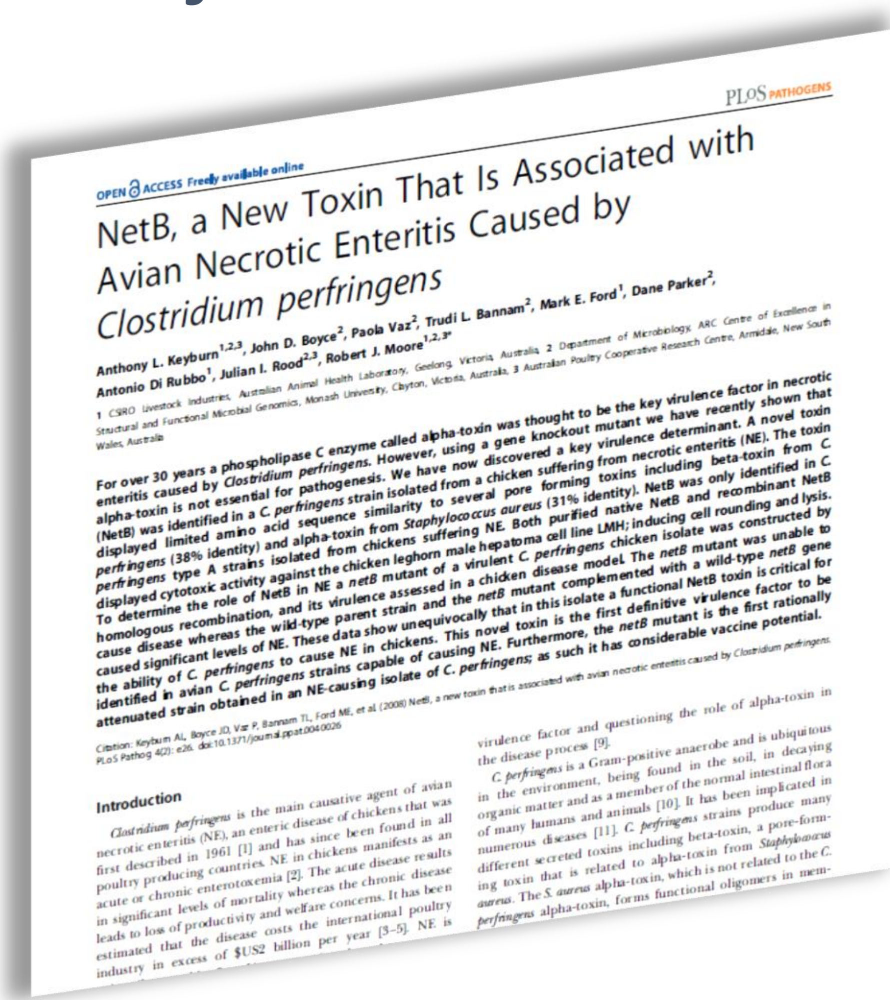
Immunization of broiler chickens against necrotic enteritis

- The challenges
- Can we immunize?
- What is the basis of immunity?
- What are the important antigens?
- What is an ideal vaccine?
- How can we deliver vaccine antigens?
- How should we test vaccines?
- Future possibilities

The challenges

- Complex disease
- Understanding the basis of protective immunity
- Immunizing in the face of maternally-derived immunity
- Identifying key antigen(s)
- Defining the best systems to test vaccines
- Safety, efficacy, robustness, cost
- Field versus lab testing

Major advance in NE research



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NetB, a New Toxin That Is Associated with Avian Necrotic Enteritis Caused by *Clostridium perfringens*

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For over 30 years a phospholipase C enzyme called alpha-toxin was thought to be the key virulence factor in necrotic enteritis caused by *Clostridium perfringens*. However, using a gene knockout mutant we have recently shown that alpha-toxin is not essential for pathogenesis. We have now discovered a key virulence determinant. A novel toxin (NetB) was identified in a *C. perfringens* strain isolated from a chicken suffering from necrotic enteritis (NE). The toxin displayed limited amino acid sequence similarity to several pore forming toxins including beta-toxin from *C. perfringens* (38% identity) and alpha-toxin from *Staphylococcus aureus* (31% identity). NetB was only identified in *C. perfringens* type A strains isolated from chickens suffering NE. Both purified native NetB and recombinant NetB displayed cytotoxic activity against the chicken leghorn male hepatoma cell line LMH; inducing cell rounding and lysis. To determine the role of NetB in NE a *netB* mutant of a virulent *C. perfringens* chicken isolate was constructed by homologous recombination, and its virulence assessed in a chicken disease model. The *netB* mutant was unable to cause disease whereas the wild-type parent strain and the *netB* mutant complemented with a wild-type *netB* gene caused significant levels of NE. These data show unequivocally that in this isolate a functional NetB toxin is critical for the ability of *C. perfringens* to cause NE in chickens. This novel toxin is the first definitive virulence factor to be identified in avian *C. perfringens* strains capable of causing NE. Furthermore, the *netB* mutant is the first rationally attenuated strain obtained in an NE-causing isolate of *C. perfringens*; as such it has considerable vaccine potential.

Citation: Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, et al. (2008) NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathog 4(2): e26. doi:10.1371/journal.ppat.0040026

Introduction

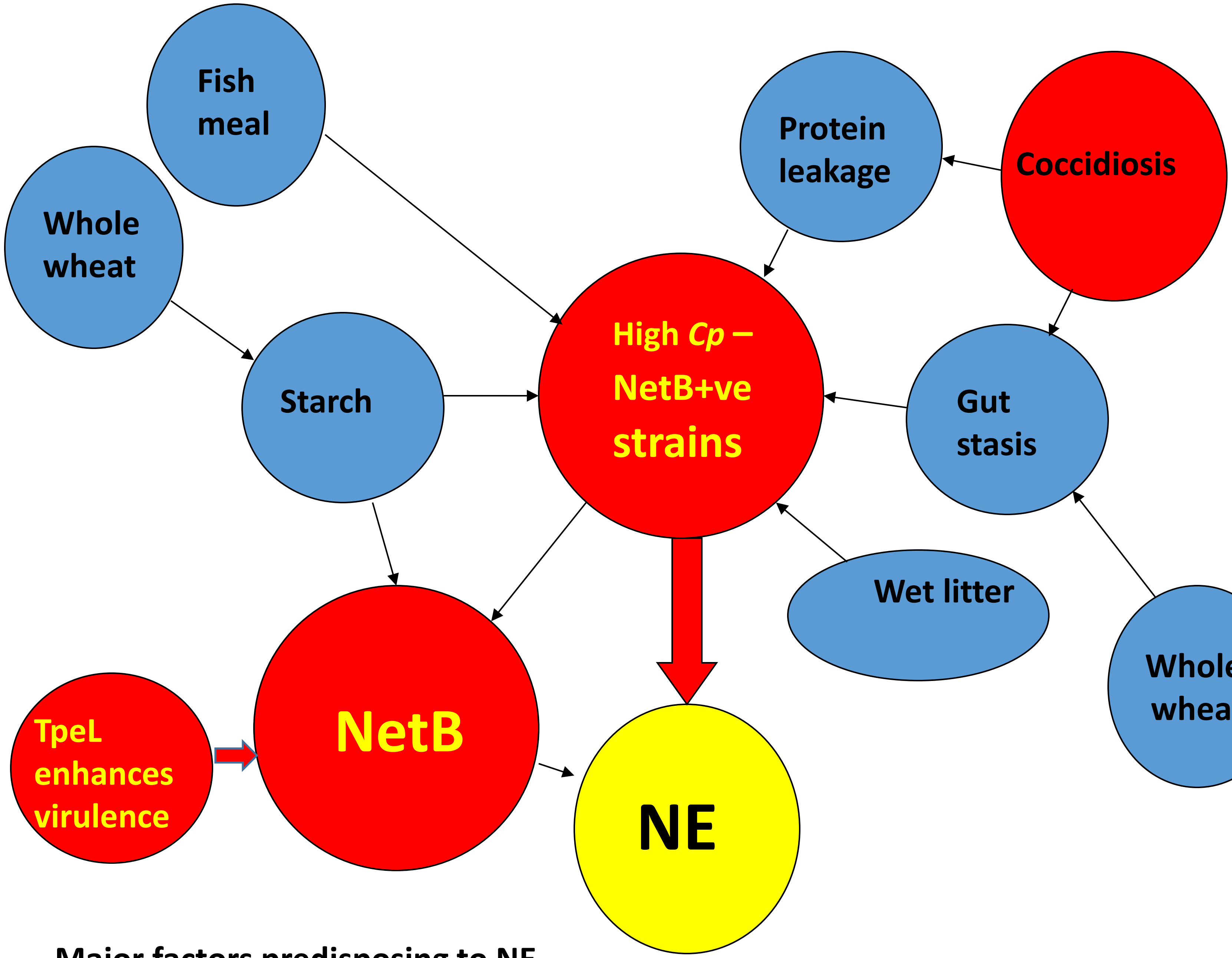
Clostridium perfringens is the main causative agent of avian necrotic enteritis (NE), an enteric disease of chickens that was first described in 1961 [1] and has since been found in all poultry producing countries. NE in chickens manifests as an acute or chronic enterotoxemia [2]. The acute disease results in significant levels of mortality whereas the chronic disease leads to loss of productivity and welfare concerns. It has been estimated that the disease costs the international poultry industry in excess of $US2 billion per year [3–5]. NE is

virulence factor and questioning the role of alpha-toxin in the disease process [9].

C. perfringens is a Gram-positive anaerobe and is ubiquitous in the environment, being found in the soil, in decaying organic matter and as a member of the normal intestinal flora of many humans and animals [10]. It has been implicated in numerous diseases [11]. *C. perfringens* strains produce many different secreted toxins including beta-toxin, a pore-forming toxin that is related to alpha-toxin from *Staphylococcus aureus*. The *S. aureus* alpha-toxin, which is not related to the *C. perfringens* alpha-toxin, forms functional oligomers in mem-

2008

- Keyburn et al. identified a novel toxin, NetB, that plays a key role in development of NE.

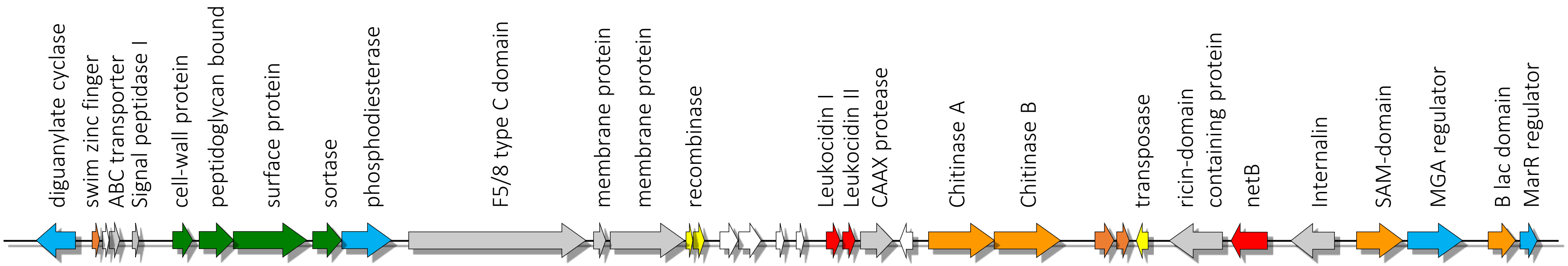


Major factors predisposing to NE

NE strains are unique

- NE genetic loci highly conserved, strongly correlated with disease
- *netB* critical, but more complex than this
- Several chromosomal loci associated with *netB*-positive isolates

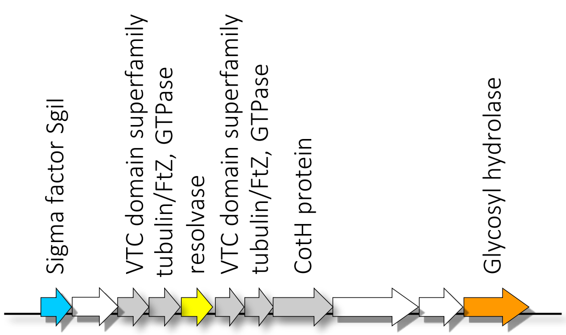
Identification of NE-specific loci



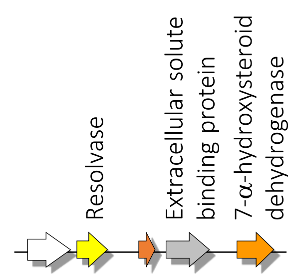
NELoc-1
(42 kb)

Putative role:

- Toxin
- Adhesion
- Regulation
- Mobilization
- Enzyme
- Other
- Plasmid
- Hypothetical protein



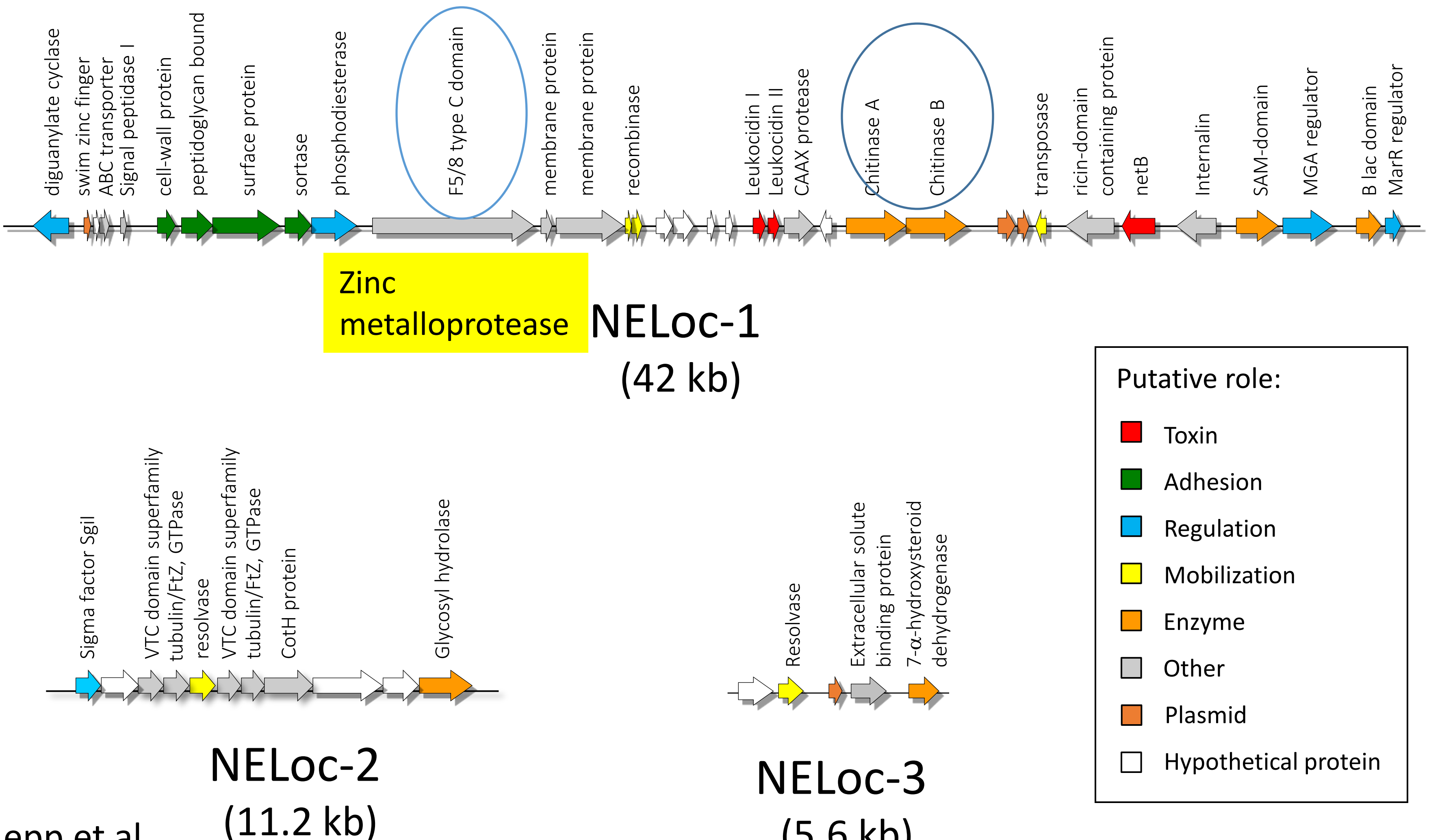
NELoc-2
(11.2 kb)



NELoc-3
(5.6 kb)

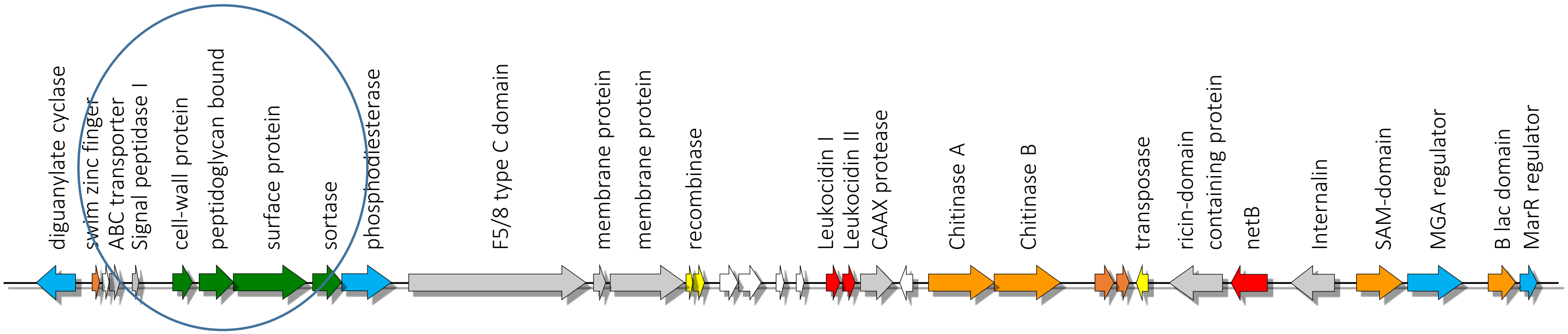
Lepp et al., 2010

NE locus 1: Mucin colonization, degradation



Lepp et al., 2010

NE locus 1: Tissue adhesion



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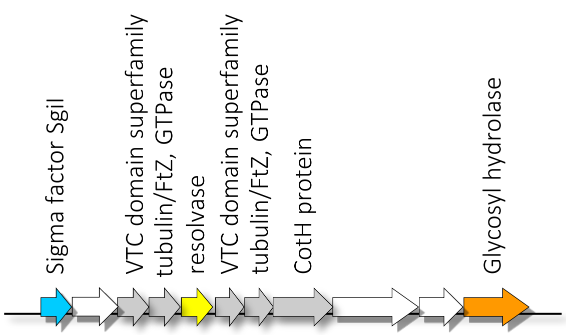

NELoc-2
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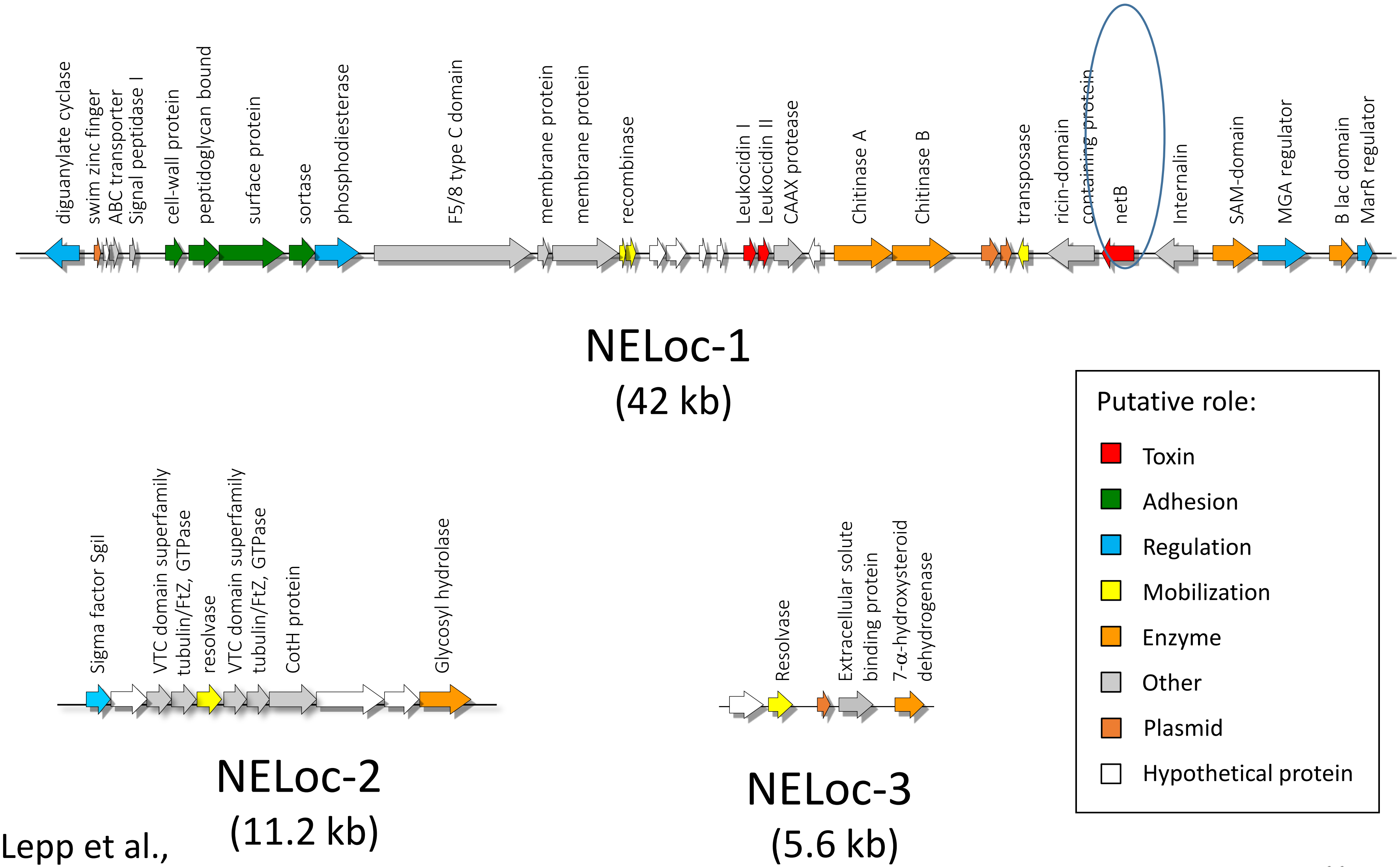
NELoc-3
(5.6 kb)

Lepp et al., 2010

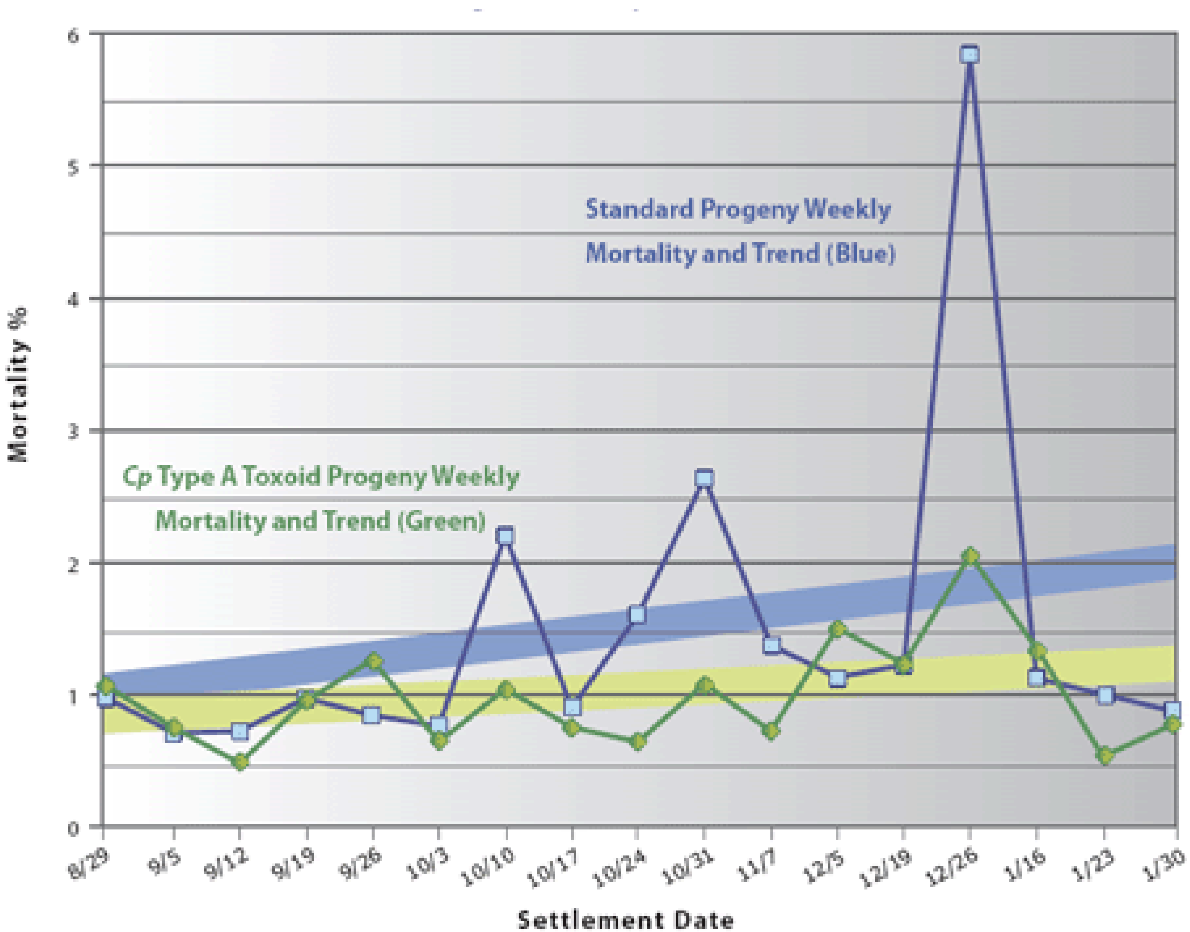
NetB toxin damage



Lepp et al., 2010

Can we immunize?

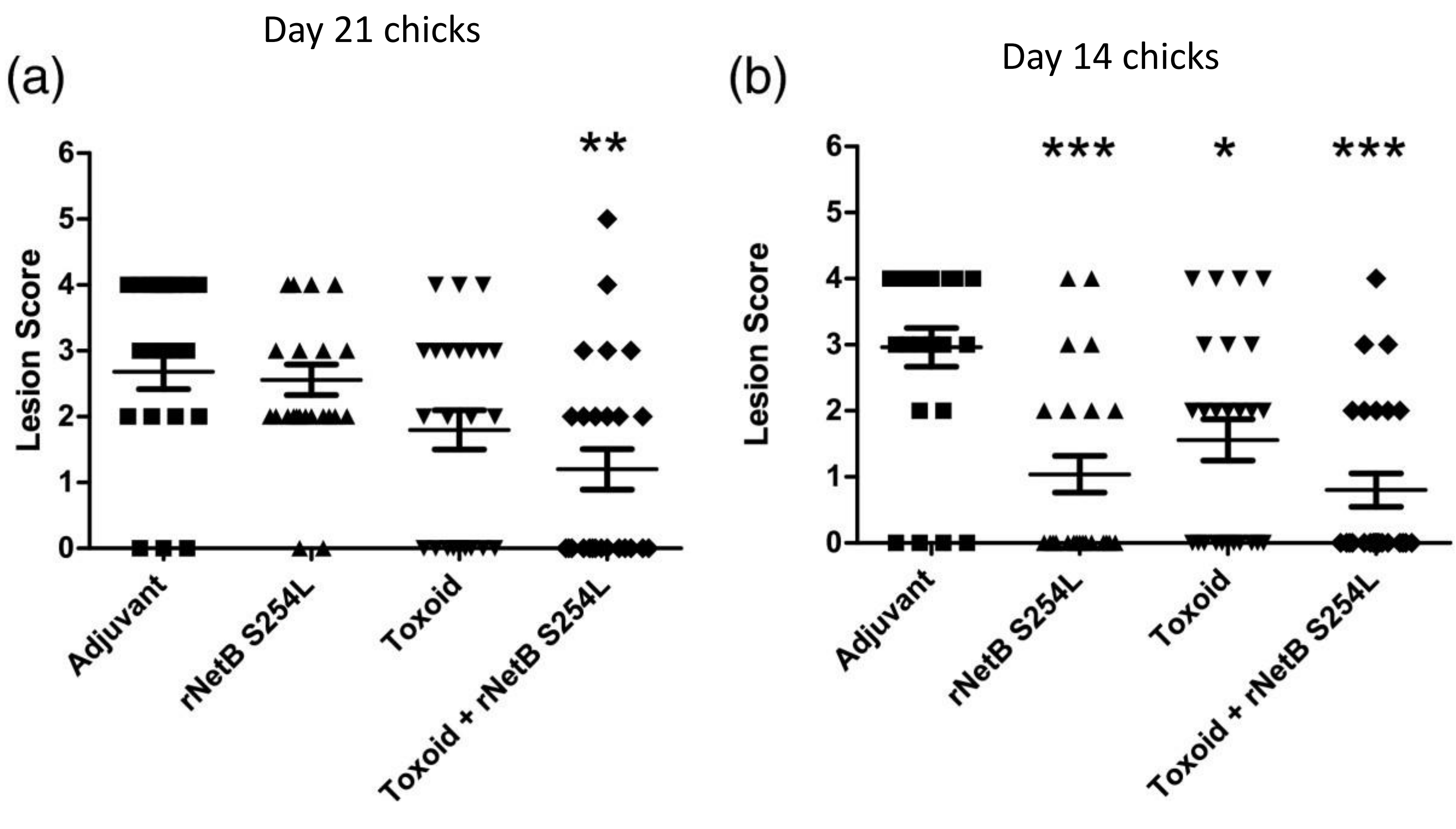
Passive immunity: NetVax vaccine for layers provides maternal antibody for broilers



"Toxoid A": supernatant antigens

Mortality in 22-28-day-old broilers

Passive immunization rNetB versus "toxoid" (Keyburn et al., 2013)



Key findings from passive immunization

- Yes, you can immunize
- Immunization with “PlcC [alpha-toxoid]” (*netB*-negative) gives good but incomplete protection
- rNetB plus “toxoid” > rNetB alone older chicks
- Useful strategy but not in later broiler production
- Need active immunization

Can we actively immunize?

Active immunization against *C. perfringens* in NE: Key lab findings

- Secreted proteins crucial
- Supernatants vary in protective ability
- Several different antigens provide reasonable protection
- Intestinal mucosal IgY >> IgA important
- Protection depends on challenge severity; system?
- Mixed antigens or chimeric proteins often better protection than individual antigens

What is the basis of immunity?

What is the basis of immunity?

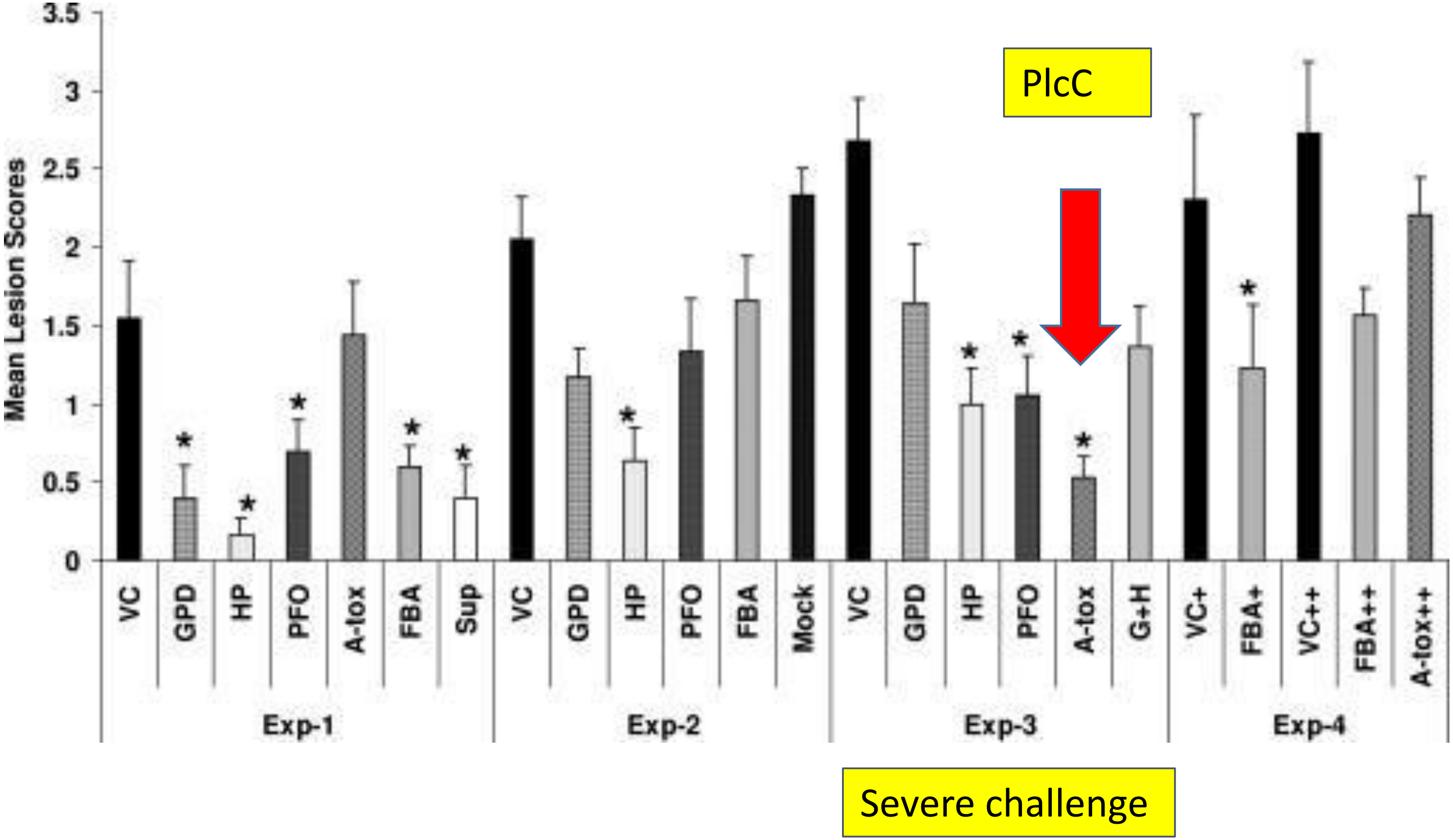
- Not understood in detail, Th2 and Th17 cytokine mediated
- Antibodies to secreted virulence factors (PlcC, NetB, zinc metalloprotease) important
- Antibodies to secreted housekeeping (“moonlighting?”) proteins important
- Many different antigens provide some protection experimentally
- Effect may be by impairing bacterial growth rather than neutralizing toxin (IgY)

Defined antigens with value in immunization against NE

Antigen	Role	Value*	Reproducibility**
Alpha toxin (PlcC)	Virulence? Phospholipase	++ …. +++	++++
NetB toxoid	Virulence	++ …. +++	++++
Zinc metalloprotease	Virulence? Mucin degradation	++ …. +++	+++
Fructose biphosphate aldolase, FBA	Housekeeping-Moonlighting? Adhesion?	+ …. +++	++++
Pilus	Virulence (collagen adhesion)	+	+
Pyruvate ferrodoxin oxidoreducatase, PFOR	Housekeeping	++	+++
Glyceraldehyde-3-phosphate oxidoreductase, GPD	Housekeeping	+ …. ++	+++
Phosphoglyceromutase, PGM	Housekeeping	++ …. +++	+
Elongation factor-Tu	Housekeeping	++	+
Endo-β-N-acetylgluosaminidase	Housekeeping	0 …. +++	+

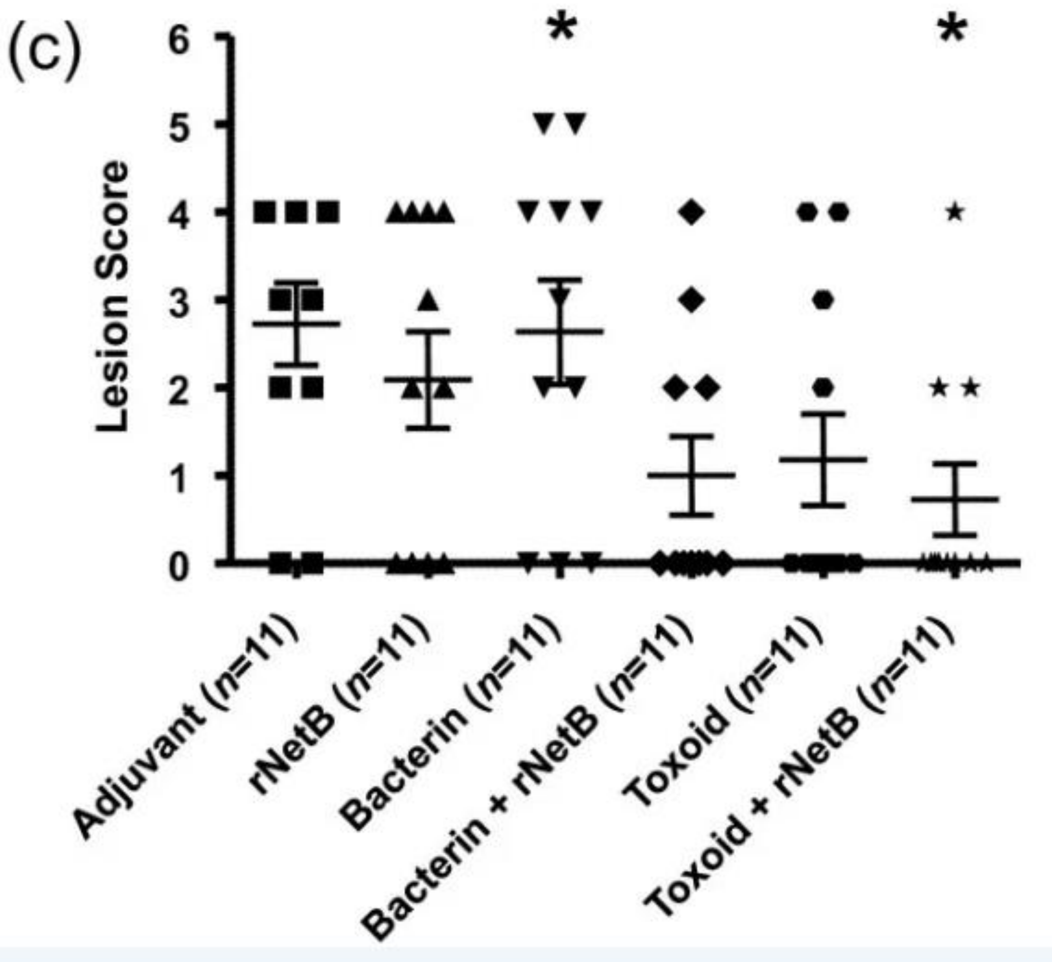
*Overall protection, + 25-49%, ++, 50-74%, +++, ≥ 75%; **, ++++, ≥ 4 studies; +++, 3 studies,

NE lesion scores in chickens immunized IM with CP proteins and different challenge severity

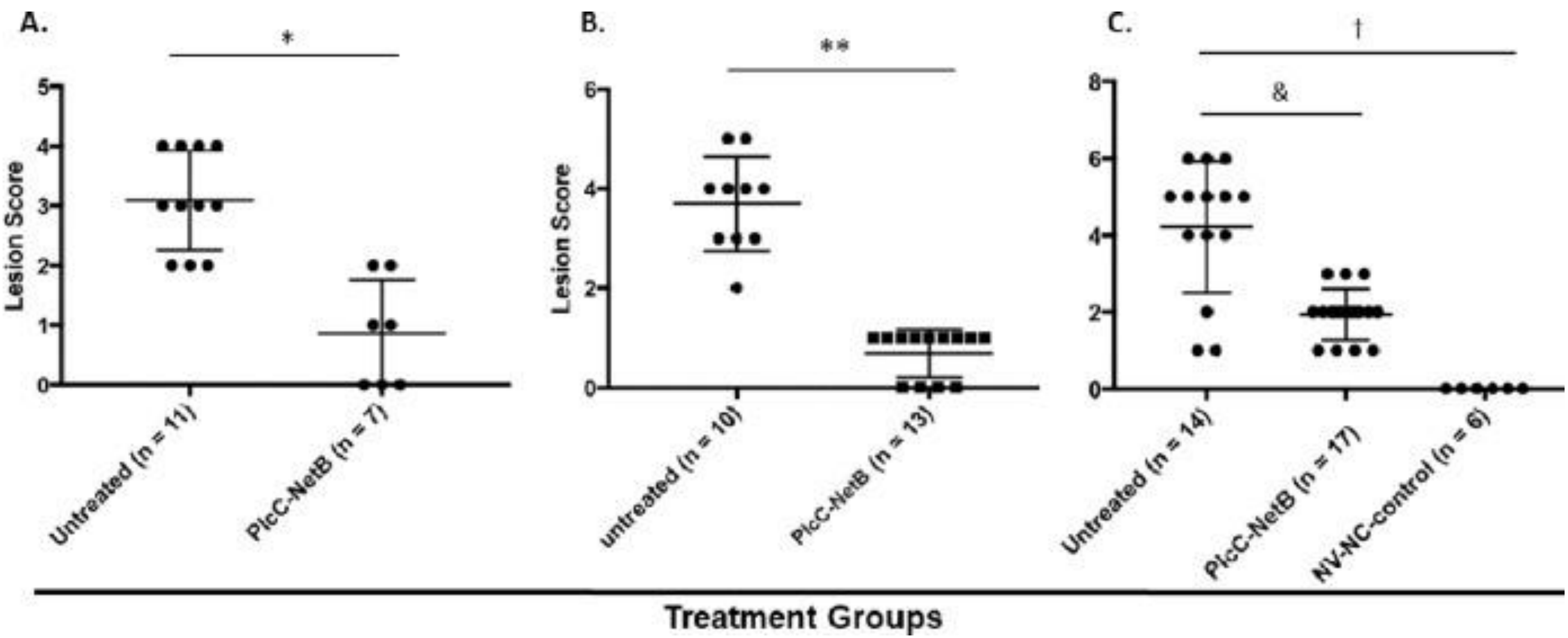


Kulkarni et al., 2007

Vaccination with recombinant NetB toxoid (Keyburn et al., 2013)



Protection of broiler chickens against NE after SC immunization with PlcC-NetB chimeric toxoid



Hunter et al., 2019a

What is an ideal vaccine?

- Safe, effective, cost-effective, profitable
- Easily administered: *in ovo* or in drinking water
- Robust under field conditions
- Local intestinal immunity important, so best if orally administered
- 100% protection

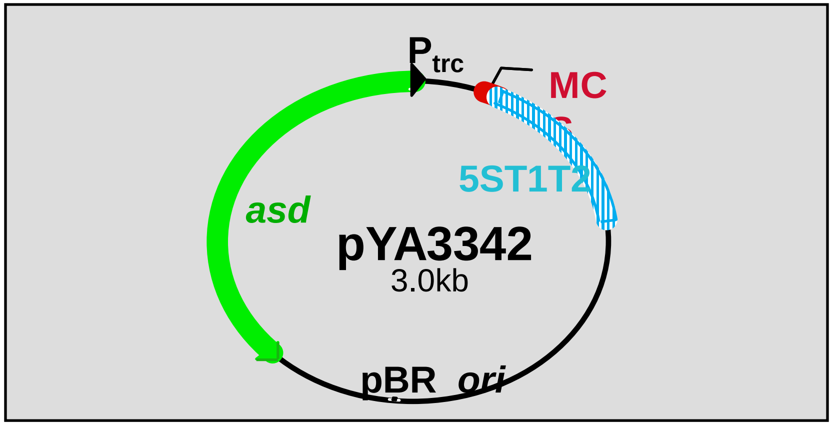
How can we deliver immunization?

Live attenuated oral *Salmonella* vectored vaccines

Salmonella vaccine vectors

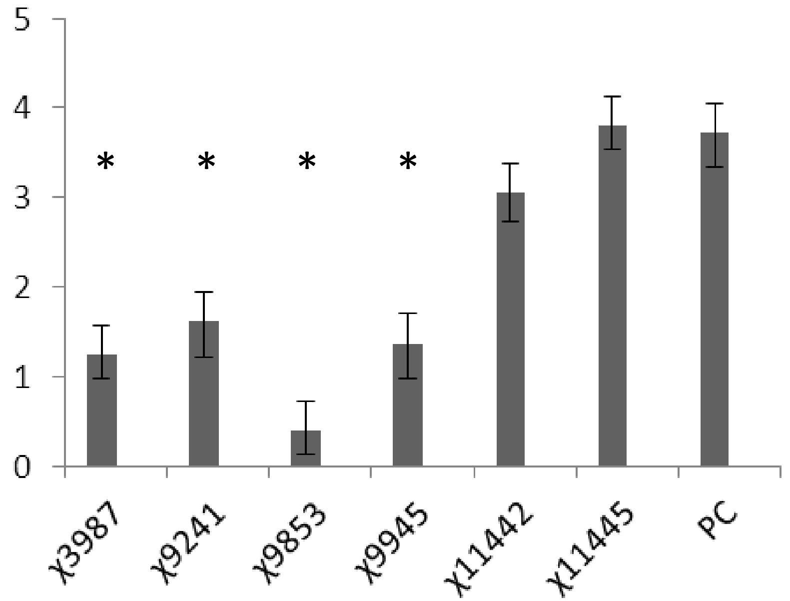
"Regulated expression" of foreign antigens; "delayed attenuation", so virulent at time of infection;

programmed lysis so disappear



Improved plasmid vectors expressing CP antigens

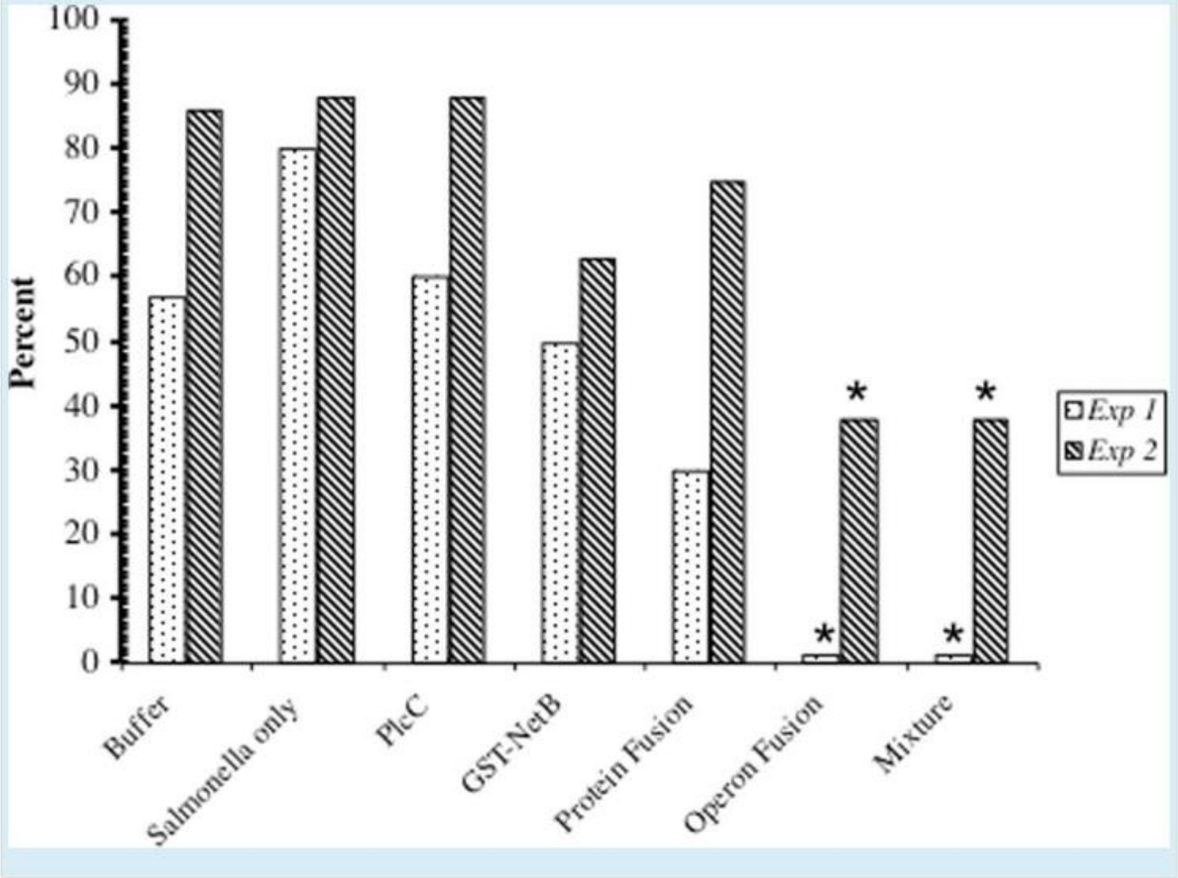
Different *Salmonella* vectors expressing PlcC have different efficacy



Intestinal lesion scores of birds immunized three times, aged 1, 7 and 14 days, with *Salmonella* vaccine vectors (χ3987, χ9241, χ9853, χ9945, χ11442, χ11445, vector only control group (χ3987), expressing the *C. perfringens* PlcC, and challenged at day 28 with *C. perfringens* CP4.

Prescott et al., unpublished

Impact of immunization with *Salmonella*-vectored PlcC and/or NetB toxoids on percentage of broiler chickens with severe lesions



Jiang et al., 2015

Mean intestinal lesion scores in broilers immunized with single *Salmonella* vaccine expressing different antigens

Group	Experiment 1	Experiment 2
Salmonella vector	2.5	3.1
Non-vaccinated controls	3.0	4.0
plcC-netB	1.5*	1.9*
plcC-fba-netB	0.8**	0.9**
fba	-	0.7**¥

Hunter et al., 2019b

Live attenuated *C. perfringens* mutants for oral vaccination? No.

Mutant	Mean + SD (% control)	Number studies
Phosphodiesterase	190 + 60	3
netB	95 + 31	3
Diguanylate cyclase	90	1
CP1-3475 ABC transporter	93	1
Zinc metalloprotease	108	1

Prescott et al., unpublished

Future possibilities

Potential avirulent live oral vaccine vectors for necrotic enteritis

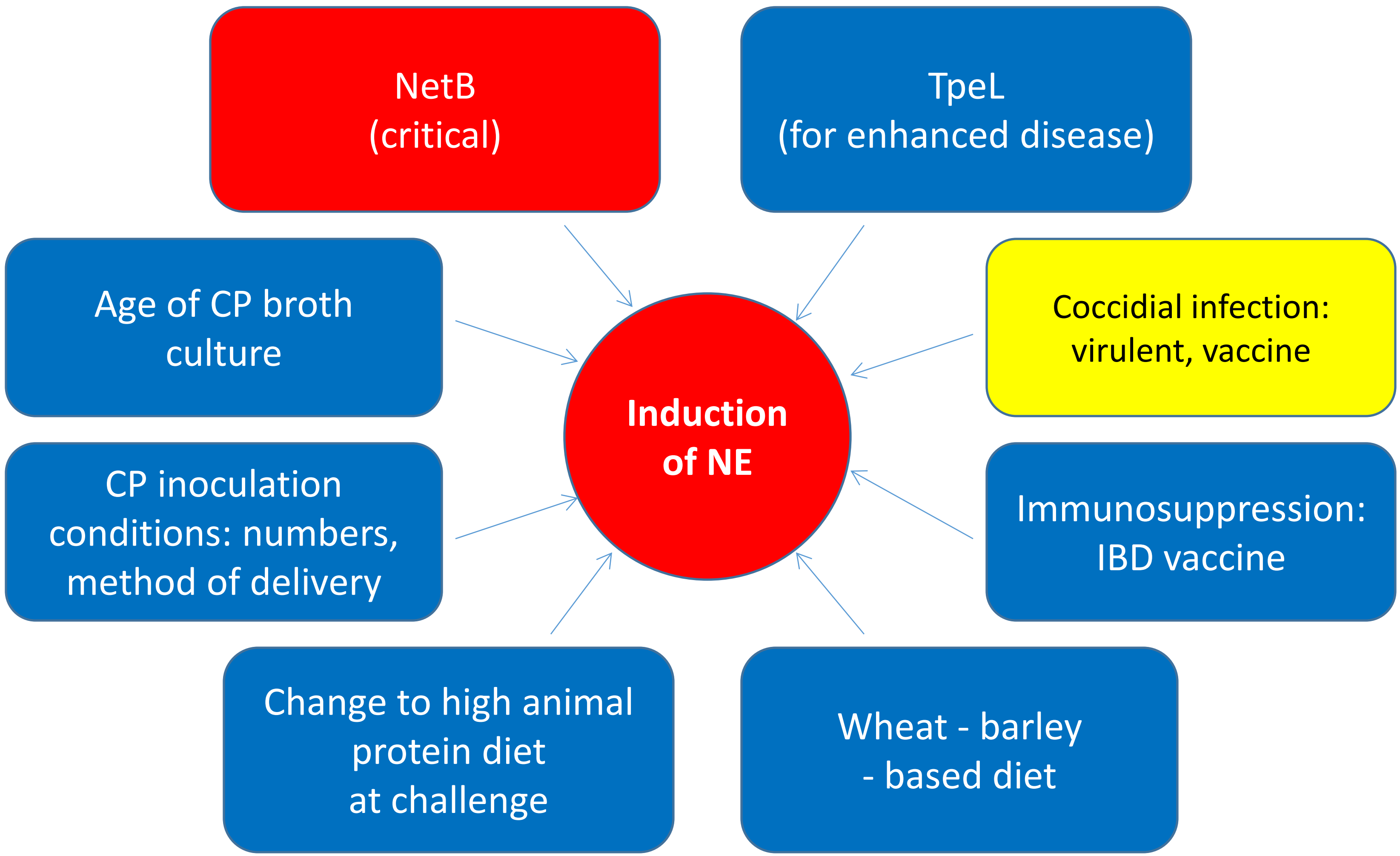
Vector	Antigen	Mouse protection	Author
Lactobacillus casei	PLC (alpha toxoid)	+++	Gao, 2019
Lactobacillus casei	PLC	+++	Alimolaei, 2017
Lactobacillus casei	PLC	+	Song, 2018
Bacillus subtilis spores	PLC	+++	Hoang, 2008

in ovo immunization?

- One study of efficacy *Eimeria* profilin and rNetB
- Partial protection against experimental NE
- Adjuvant important
- Far more work needed

How should we test vaccines experimentally?

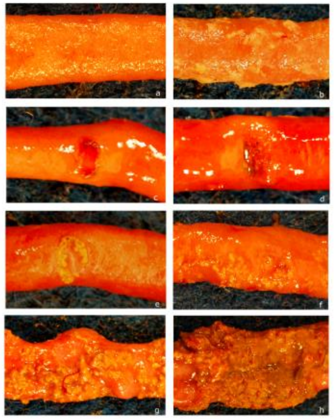
Reproducing necrotic enteritis experimentally



Shojadoost et al., 2011

Assessment of vaccine efficacy: Important issues

- No standard model to reproduce NE: Does *Eimeria* bias results?
- No challenge strain(s) criteria
- No standard assessment system
- No standard challenge severity
- No "gold standard" vaccine comparison



6 point NE scoring system:
Keyburn et al., 2008

The future

- Immunization has promise in reducing NE
- Very useful adjunct in control
- Oral attenuated *Salmonella* with “mixed” antigens promising
- Need for field testing attenuated *Salmonella* vaccines
- Explore other avirulent vaccine vectors
- *in ovo* immunization plus oral vaccine boost?

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