Towards in vitro methods for potency testing of fish vaccines

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Introduction

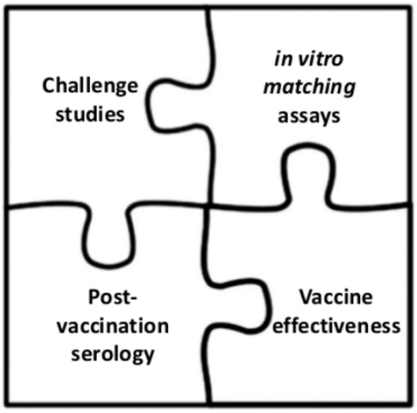
Background and motivation for developing *in vitro* methods potency testing for fish vaccines

Definitions of *in vitro* methods

Regulations

Examples – feasibility

Way forward – next steps



Background

Fish used in experimental studies in Norway in 2014 was 5.5 million

- 4.9 million were accounted for in 3 different field experiments
- 180 000 fish used in experiments *imposing pain on the animals*

Use of fish for potency testing

- Classical testing methods based on vaccination and challenge
- Mortality as end-point
- Humane end-points

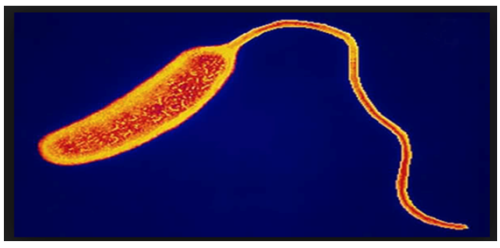
Alternatives

- *In vitro* potency studies
- In the spirit of the 3R´s

In vitro potency – some considerations

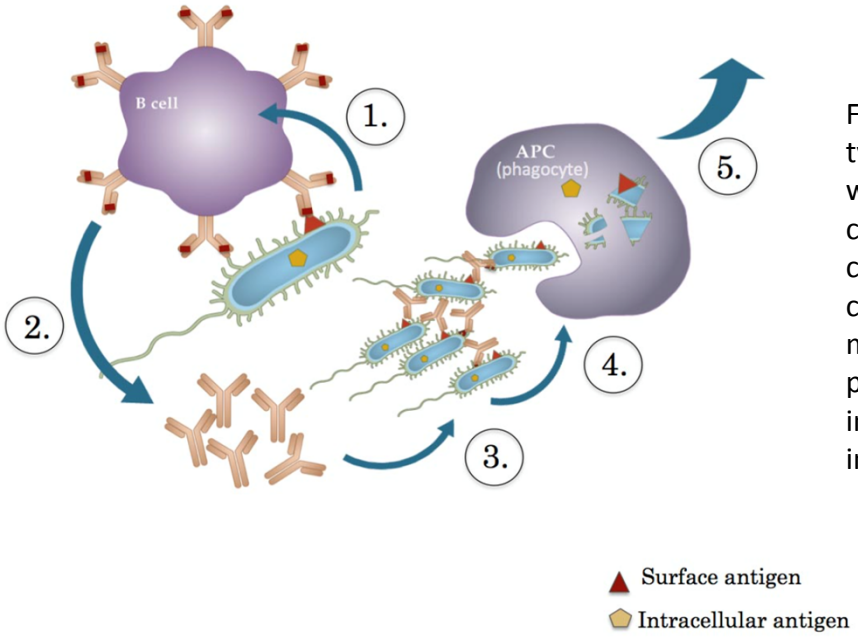
Classical approach

- Based on circulating antibodies
- Build correlation with in vivo challenge studies (during development) using dose-response studies
- Purpose – define a cut-off that separates potent from sub-potent batches as defined during development



Should the antibodies react with the protective antigen ?

- Not necessarily as long as the method used can be sufficiently defined
- Antibodies against bacteria (and also viruses) that are important for protection against infection or disease would react with surface antigens of the bacterium – a flagellum as a typical one
- For many (fish) pathogens, the protective antigen(s) has/have not been defined



Further, antibodies typically "collaborate" with soluble components like complement and circulating cells like macrophages/ phagocytes to kill / inactivate the intruder

In vitro potency – additional considerations

Species

- Would it have to be target species?
  - Not necessarily as long as a correlation can be defined
  - There will be practical challenges with species being very small
  - And complexity of the immune system would also have to be considered (and how this can impact)

Examples

- I will present two examples related to developing in vitro methods for vaccine potency testing of furunculosis vaccines in salmon
- And some recent findings on *Y. ruckeri* in trout
- This shows a step-wise approach towards establishing a
  - correlation between antibody responses and vaccine potency
  - and the use of antigen content estimation as a proxy of vaccine potency

Contents lists available at SciVerse ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Development of an antibody ELISA for potency testing of furunculosis (*Aeromonas salmonicida* subsp *salmonicida*) vaccines in Atlantic salmon (*Salmo salar* L)

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[c] Norwegian School of Veterinary Science, P.O. Box 8146 Dep, 0033 Oslo, Norway
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Biologicals 40 (2012) 67-71

Purpose of this study

- [understand] how the antibody response against *A. salmonicida* develops with time in Atlantic salmon held in different water temperatures
- to which degree it [*antibody levels*] correlates with protection following *in vivo* challenge
- optimise and standardise an antibody-based ELISA potency test procedure

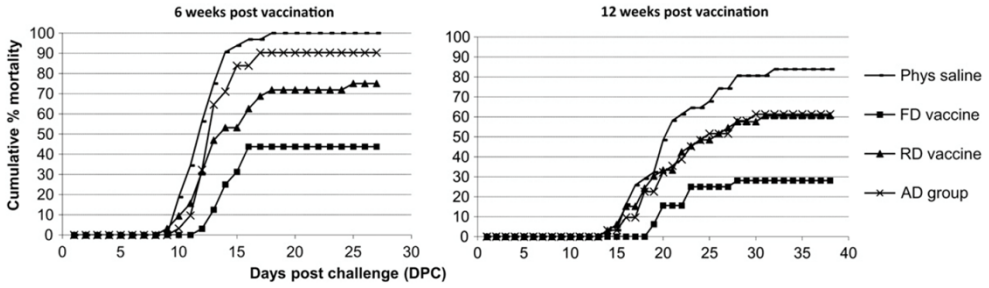
- deviation from EP requirements
  - Challenge carried out by cohabitation

Materials and methods

- used two vaccine doses (Full dose (FD) and reduced dose (RD – 1/20 of FD)) + adjuvant control (AD)
- sampled serum over time (time course study); 3, 6, 9, and 12 weeks post vaccination
- challenge at 6 and 12 weeks post vaccination

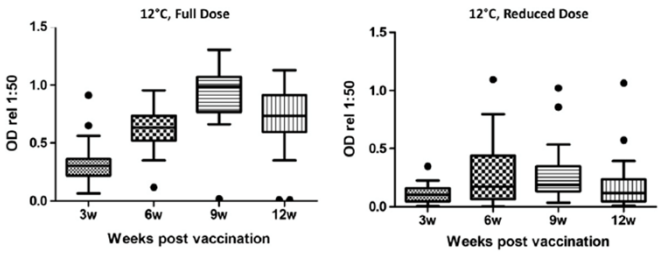
Results

A.B. Romstad et al. / Biologicals 40 (2012) 67–71



ELISA analysis

A.B. Romstad et al. / Biologicals 40 (2012) 67–71



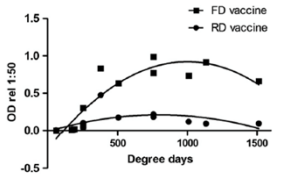


Fig. 3. Antibody response against sonicated *Aeromonas salmonicida* related to degree days in fish vaccinated with Full Dose (FD) vaccine (100% *A. salm* antigen content compared to commercial vaccines)and Reduced Dose (RD) vaccine (5% *A. salm* antigen content compared to commercial vaccines). Degree days are immunisation temperature in degree Celsius multiplied with number of immunisation days at that temperature. ■ is Full Dose (FD) vaccine. ● is Reduced Dose (RD) vaccine. The polynomial trend line represents the trend between the observations of each vaccine. OD rel 1:50 is measured optical density (OD) at 450 nm for each fish is divided on the optical density of the positive control on each plate. Serum dilution 1: 50. All samples in duplicate wells. $N = 20$ fish per group.

Dose - effect

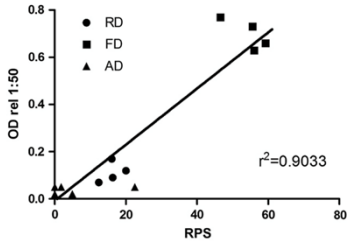


Fig. 4. Relative Percent Survival (RPS) versus median Relative antibody response against sonicated *Aeromonas salmonicida* (*A. salm*). Sampling and challenge at 6 and 12 weeks post vaccination in the groups immunised > 500° days are included in the figure. ■ is Full Dose (FD) vaccine (100% *A. salm* antigen content compared to commercial vaccines). ● is Reduced Dose (RD) vaccine (5% *A. salm* antigen content compared to commercial vaccines). ▲ is the control groups (the adjuvant group and the physiological saline group). Spearman's Rank Correlation (non-parametric), $r = 0.81 (p < 0.01)$, Pearson's correlation, $r = 0.94$ ($p < 0.01$), r^2(regression coefficient) = 0.9. OD rel 1:50 is measured optical density (OD) at 450 nm for each fish is divided on the optical density of the positive control on each plate. Serum dilution 1: 50. All samples in duplicate wells. Relative Percentage Survival (RPS) calculated across parallel tanks according to Amend (1981).

- We obtained a very nice relationship between antigen dose and RPS in this experiment
- Caution should be exercised – no dose-response study as such

Conclusions

- The results show that an ELISA assay for serum antibody level against *A. salmonicida* correlates well with protection after an immunisation period of approximately 500 degree days
- The assay gave a significant difference between a full dose (FD) and a reduced antigen dose (RD)

Vaccine 31 (2013) 791–796

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Antibody responses correlate with antigen dose and *in vivo* protection for oil-adjuvanted, experimental furunculosis (*Aeromonas salmonicida* subsp. salmonicida) vaccines in Atlantic salmon (*Salmo salar* L.) and can be used for batch potency testing of vaccines

Anne Berit Romstad[a,e,*], Liv Jorun Reitan[b], Paul Midtlyng[c], Kjersti Gravningen[d], Øystein Evensen[c]

Aim

- evaluate the ability of different vaccine evaluation methods to identify sub-potent furunculosis vaccines, using *ELISA as in vitro assay*

- *Deviation from EP:* cohabitation challenge but injection challenge was also included

Antigen/vaccine preparations

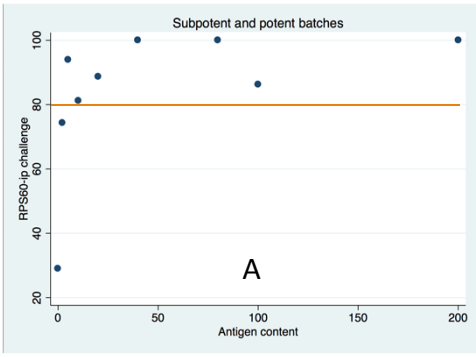
Vaccine	
Antigen	Antigen content
A. salmonicida virulent strain	0%
	2%
	5%
	10%
	20%
	40%
	80%
	100%
	200%
A. salmonicida avirulent strain	5%
	100%
Saline control	0%

Fine-tuned the antigen dosage – dose-effect study

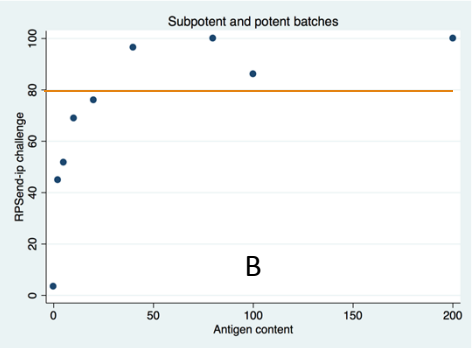
Results

		Intra peritoneal challenge			Cohabitation challenge		ELISA
	Antigen content	CM%	RPS_{60} [a]	RPS_{end} [b]	CM%	RPS_{end} [b]	Rel OD[c]
A salmonicida	0%	87.5	29.1	3.4	53.1	10.5	0.09
	2%	50.0	74.3	44.8	34.4	42.1	0.27
	5%	43.8	93.9	51.7	53.1	10.5	0.25
	10%	28.1	81.3	69.0	40.6	31.6	0.41
	20%	21.9	88.7	75.9	15.6	73.7	0.44
	40%	3.1	100.0	96.6	6.3	89.5	0.64
	80%	0	100.0	100.0	12.5	78.9	0.80
	100%	12.5	86.2	86.2	3.1	94.7	0.73
	200%	0	100.0	100.0	6.3	89.5	0.84
A layer neg	5%	75.0	54.8	17.2	50.0	15.8	0.22
	100%	68.8	58.2	24.1	59.4	0	0.43
Control	0%	90.6	–	–	59.4	–	0.07

Antigen content and challenge models

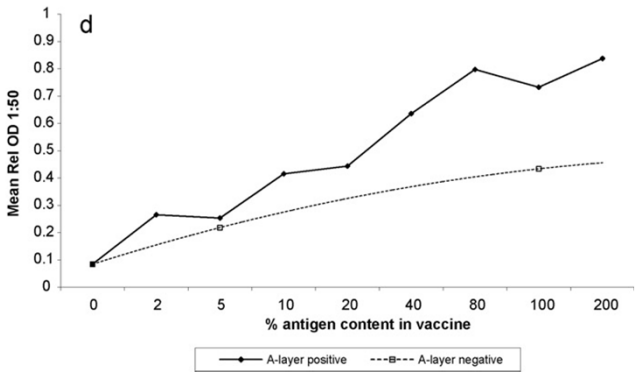


In (A) we see that vaccines with antigen content as low as 5% of standard will come out as potent using RPS_{60} evaluation



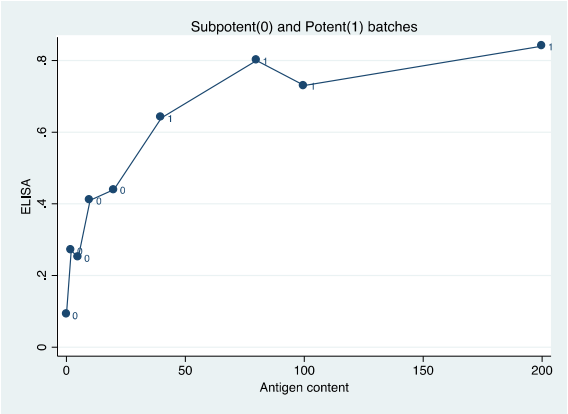
Estimating RPS at end of challenge in (B) only vaccines with 40% of standard ag content will pass (RPS>80)

Antigen content and ELISA (OD)

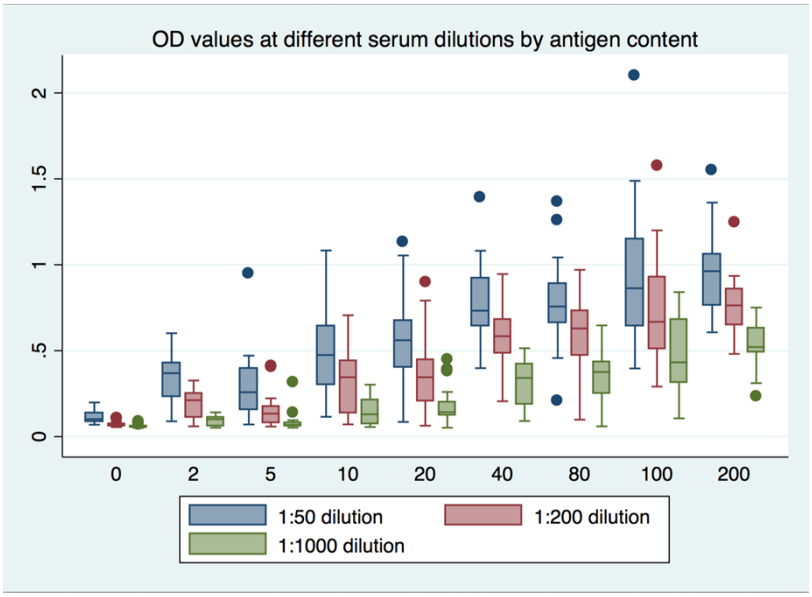


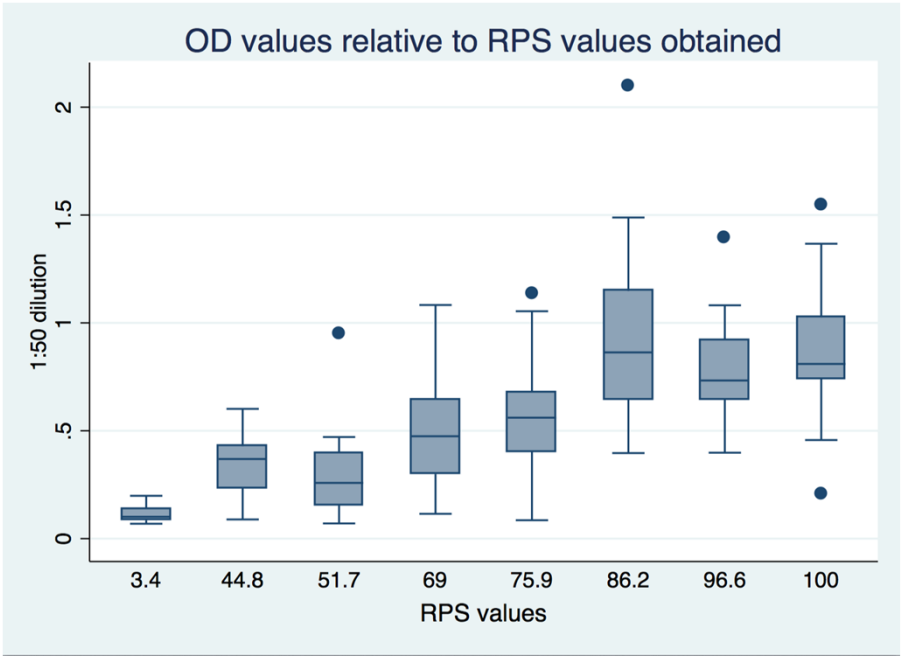
OD relative to antigen and potency

- Here the OD ELISA values have been plotted against antigen content and at the same time result from potency test – endpoint mortality by injection challenge
- As seen – OD values above 0.6 (1:50 dilution) is found for potent vaccines, i.e. vaccines that meet the RPS≥80 criterion

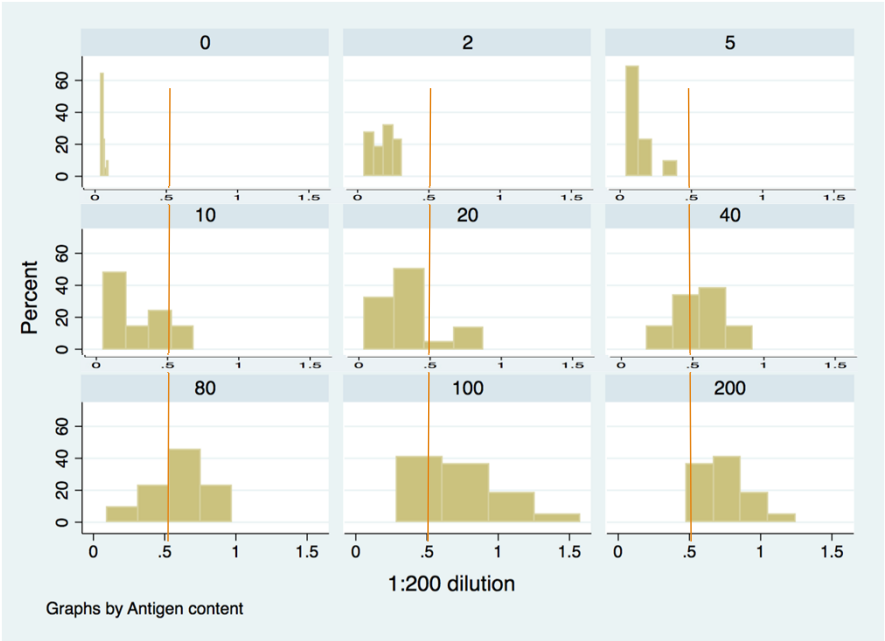


Antibody levels by dilution and antigen content





Distribution of responders in different vaccine groups



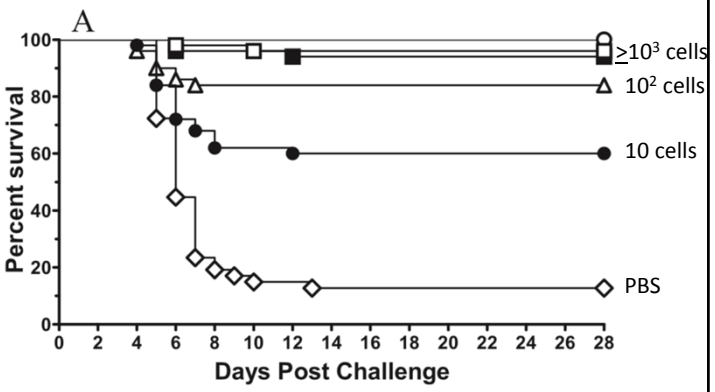
Conclusions

- There is a close correlation between the antigen dose and the antibody response against *Aeromonas salmonicida* as measured by ELISA
- Close correlation between the antibody response and protection for both i.p. and cohabitation challenge models
- ELISA method identified sub-potent batches not identified when using an RPS_{60} assessment protocol
- In vitro methods based on antibody responses for furunculosis vaccine potency testing carry potential as a batch release method considering 3R's principles and animal welfare

Correlation to antigen dose and test of protective antigen (*Y. ruckeri*)

Bacterins

- Vaccines were delivered by IP injection at indicated concentrations:
  - closed diamond, 10^8 cells/fish
  - open circle, 10^6 cells/fish
  - closed square, 10^4 cells/fish
  - open square, 10^3 cells/fish
  - open triangle, 10^2 cells/fish
  - closed circle, 10 cells/fish
  - open diamond, PBS mock
- Rainbow trout were challenged by immersion exposure to 1.0×10^9 *Y. ruckeri* for 1 h in a static bath at 28 dpv

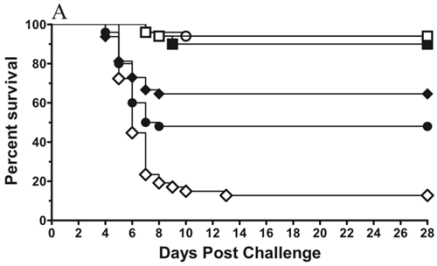


B

Vaccination Group (cells/fish)	10^8	10^6	10^4	10^3	10^2	10	PBS
Survival (%)	100	100	94	96	84	60	13

Protective antigen - LPS

- Effect of vaccination with purified *Y. ruckeri* LPS on mortality induced by exposure to *Y. ruckeri*
- Rainbow trout were challenged by immersion exposure to 1.0×10^9 *Y. ruckeri* for 1 h in a static bath at 28 dpv.
- LPS was delivered by IP injection
- Panel A shows survival curves for each treatment
- Panel B the percent mortality at day 28 is shown for each treatment



B

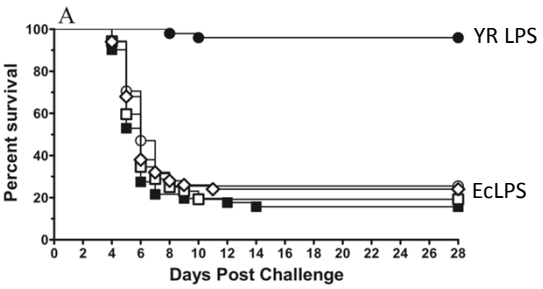
LPS (ng/fish)	100	10	1	0.1	0.01	PBS
Survival (%)	94	90	94	65	48	13

Welch & LaPatra 2016

Specificity of the response elicited

- Specificity of immune responses was tested by injecting fish with *E coli* LPS (EcL; purified) at concentrations shown (heterologous)
- *Y ruckeri* (YR) LPS was given at 10 ng/fish

A



B

	E. coli			*Y. ruckeri*	
LPS (ng/fish)	1000	100	10	10	PBS
Survival (%)	26	16	19	95	24

Conclusions

- Study shows that protection correlates with antigen content
- Protective antigen is LPS

THANK YOU FOR YOUR ATTENTION

Onset of the antibody response to bacterial vaccine antigens in Atlantic salmon

Paul J. Midtlyng[1], Anne Ramstad[2], Liv Jorun Reitan[3]

[1] *NMBU School of Veterinary Medicine, Oslo*

[2] *VESO Vikan, Namsos*

[3] *Norwegian Veterinary Institute, Oslo*

ECVAM meeting on fish vaccines, Ispra, January 2008

Some 20 recommendations for Refinement, Replacement and Reduction were made and argued

Only few of these have been pursued by industry or regulatory bodies for real-life implementation

Lack of instruments or lack of will to make change happen?

Biologicals 39 (2011) 117–128

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Meeting report

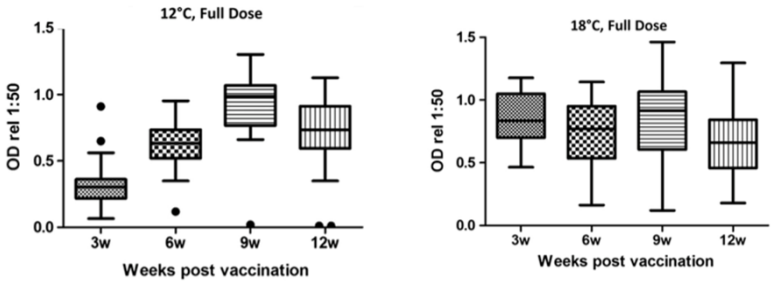
Three Rs approaches in the production and quality control of fish vaccines

Paul J. Midtlyng [a,*], Coenraad Hendriksen [b], Elisabeth Balks [c], Lukas Bruckner [d], Lawrence Elsken [e], Øystein Evensen [f], Kjetil Fyrand [g], Allison Guy [h], Marlies Halder [i,*], Penny Hawkins [j], Gunn Kisen [k], Anne Berit Romstad [l], Kira Salonius [m], Patrick Smith [n], Lynne U. Sneddon [o]

«(3R) talk is cheap»
Recommending others to observe 3R is even cheaper (?)

Establishment of an Ab-based batch test: temperature dependence of the response

EP recommended batch tests (immunisation+ challenge) take 2-3 months



From: Romstad AB, Reitan LJ, Midtlyng P, Gravningen K, Evensen Ø; Biologicals 40 (2012) 67-71.

«Antibody ELISAs - an alternative to challenge trials for batch potency testing of fish vaccines»

- NRC funded project 2015-2016
- School of Veterinary Medicine NMBU; VESO Vikan, Norwegian Veterinary Institute
- Work plan:
  - WP1:Kinetics of the antibody responses against bacterial antigens at water temperatures above 12°C…, and the ability of Ab ELISA to reveal subpotent batches
  - WP2: Quantifying the animal welfare gain by replacing challenge tests with euthanasia and blood sampling

Design of vaccination trials

V-3447

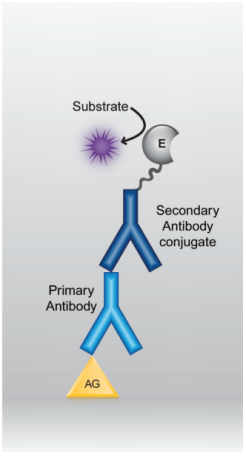
- Vaccine A (6 antigens, oil adjuvanted)
- Water temperature 12˚C or 15˚C (parallel tanks)
- Plasma taken 0,3,4,5,6 and 9 weeks post vacc. (wpv)

V-3570

- Vaccine B (6 antigens, oil adjuvanted) + unvaccinated controls
- Water temperature 15˚C
- Plasma taken 0,3,4,5,6 weeks post vaccination

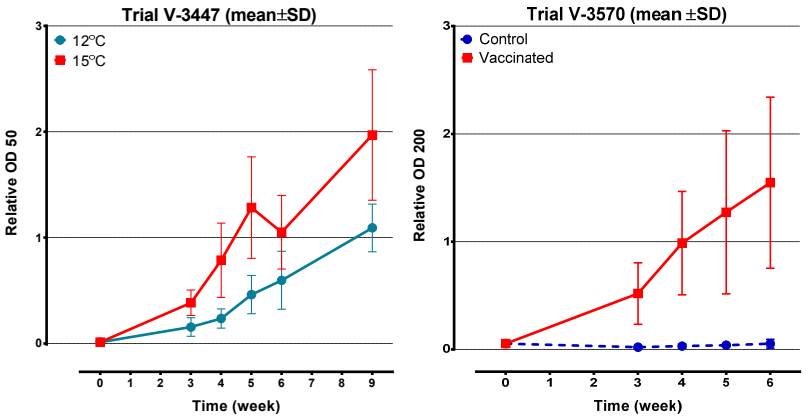
ELISA method

- Capture antigens: sonicated bacteria (Norwegian strains)
- OD readings normalised against a positive pool run on every plate (relative OD)
- Average of duplicate wells

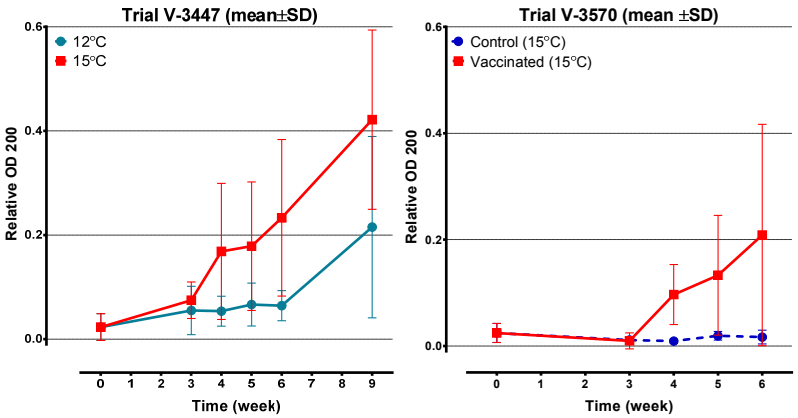


Erdal and Reitan 1992; Fish & Shellfish Immunology 2, 99–108.
Romstad et al. 2012; Biologicals 40 (2012) 67-71.
Løvoll et al. 2009; Fish & Shellfish Immunology 26, 877-84.

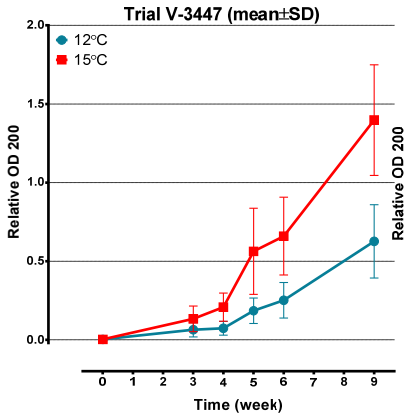
Ab response kinetics to *A. salmonicida*

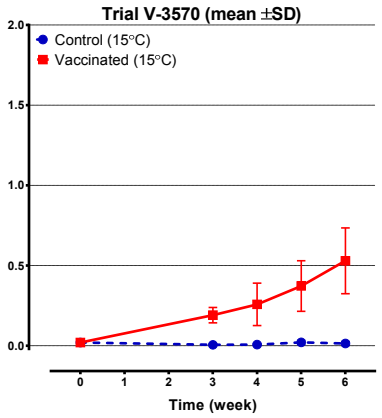


Ab response kinetics to *V. salmonicida*

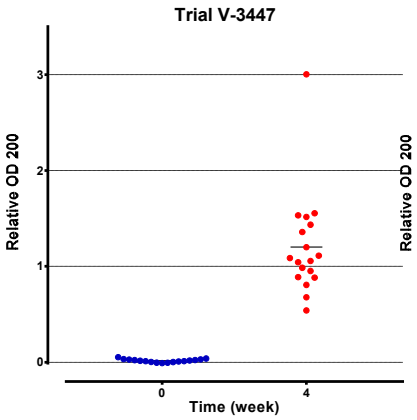


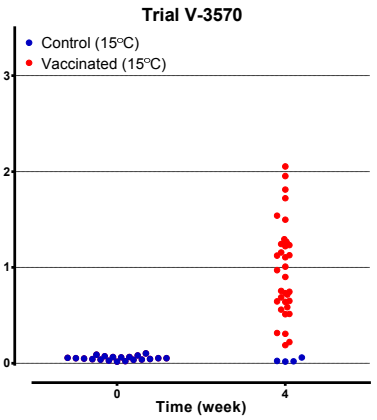
Ab response kinetics to *M. viscosa*



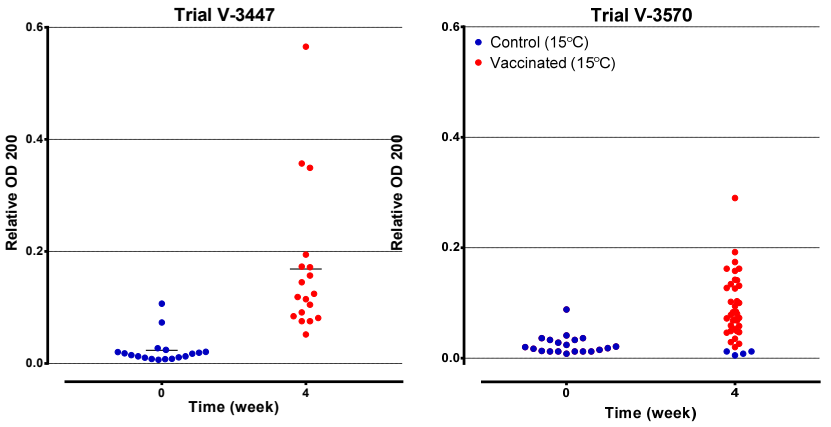


Individual fish responses to *A. salmonicida*

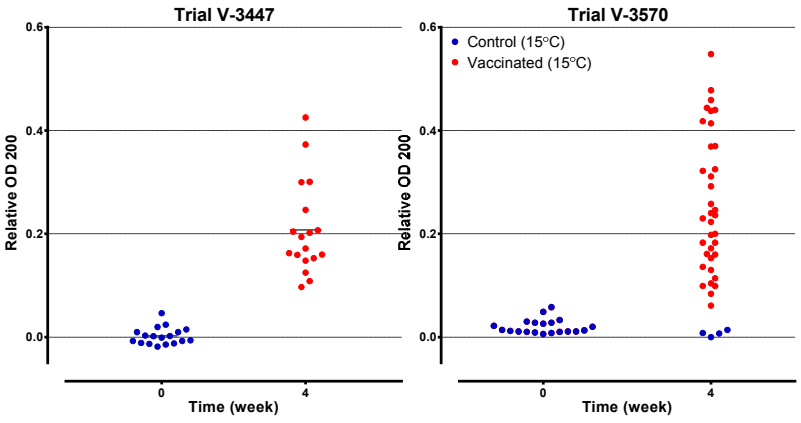




Individual fish responses to *V. salmonicida*



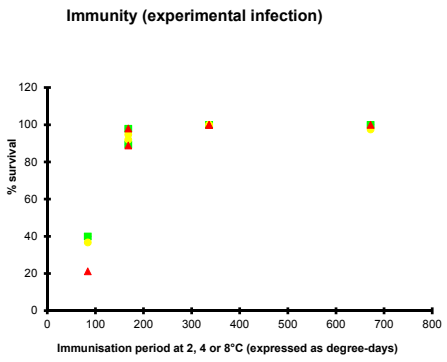
Individual fish responses to *M. viscosa*

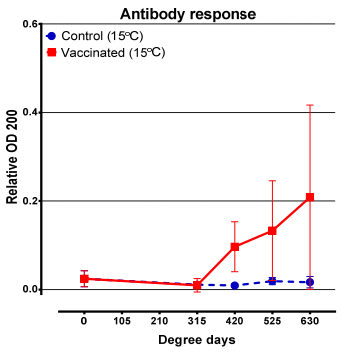


Remaining challenges and work

- Magnitude and specificity of the Ab response to *Vibrio salmonicida* and other *Vibrio* antigens
- WP1: Vaccination experiment using defective vaccine formulation(s)
- WP2: Comparison of brain serotonin responses in vaccinated salmon subjected to or relieved from experimental bacterial challenge
- WP2: Proposing a model for retrospectively assessing and documenting the welfare outcome of vaccine batch potency tests

Development of immunity and antibody response to Vibrio salmonicida





Bead-based multiplex ELISA

Initial development of a novel, bead-based format for Ab ELISA of plasma samples from Atlantic salmon is being carried out

- Assessment of dynamic assay range for fish pathogens
- Investigations into the feasibility for industrial routine application (multiplexing)

See poster by Hege Lund, Paul J. Midtlyng and Anne Storset, NMBU

Acknowledgements

We thank:

- Ms. Mariann Finnanger and VESO Vikan staff for conduct of vaccination experiments and samplings
- Ms. Karen Bækken Soleim for ELISA analyses
- Pharmaceutical companies for donation of fish vaccines