Evaluation of *in vitro* mutagenicity assays

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Objectives

Mutagenicity Assays:

- Counts, Proportions as single endpoints
- Dose-Response setting
- Dose-Response shape rarely known
- Reasoning for significance and biological relevance

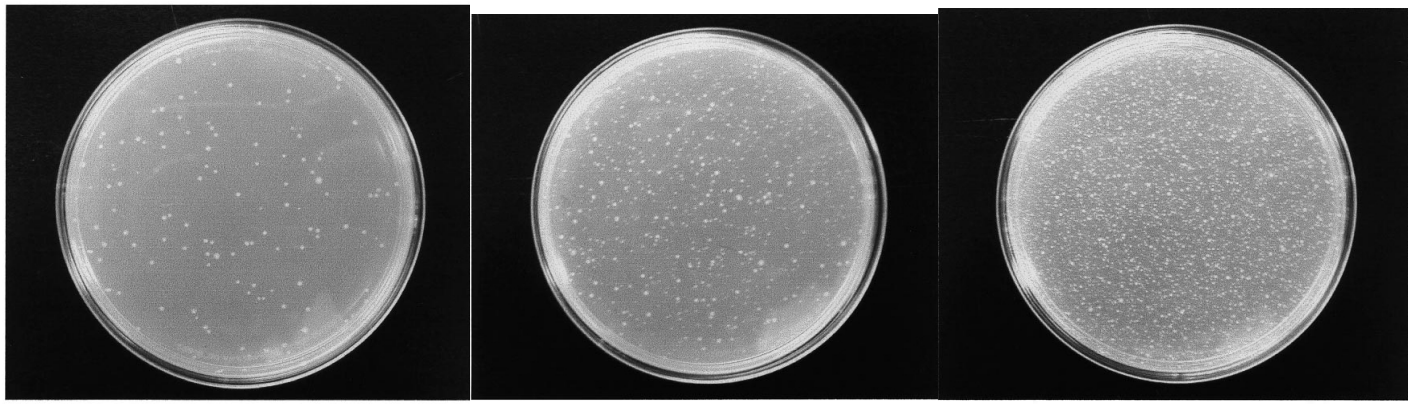
Proposed Method:

- Negative-Binomial, Beta-Binomial, or Quasi-Likelihood Models
- Ratio-to-control comparisons on trend by Williams or Williams protected contrasts
- Calculation of simultaneous confidence intervals

Examples for: Ames Salmonella Assay, *in vitro* Micronucleus Assay

Ames Salmonella Assay

- Altered *Salmonella* strains are exposed to different concentrations of a chemical to test for toxicity
- The altered *Salmonella* require histidine for growth
- Mutation can cause the bacterial strains to grow without histidine
- The number of bacterias per plate indicate the rate of mutation caused by the chemical



[Mortelmans, Zeiger 2000]

Quinoline data (TA98, rat liver homogenate S9)

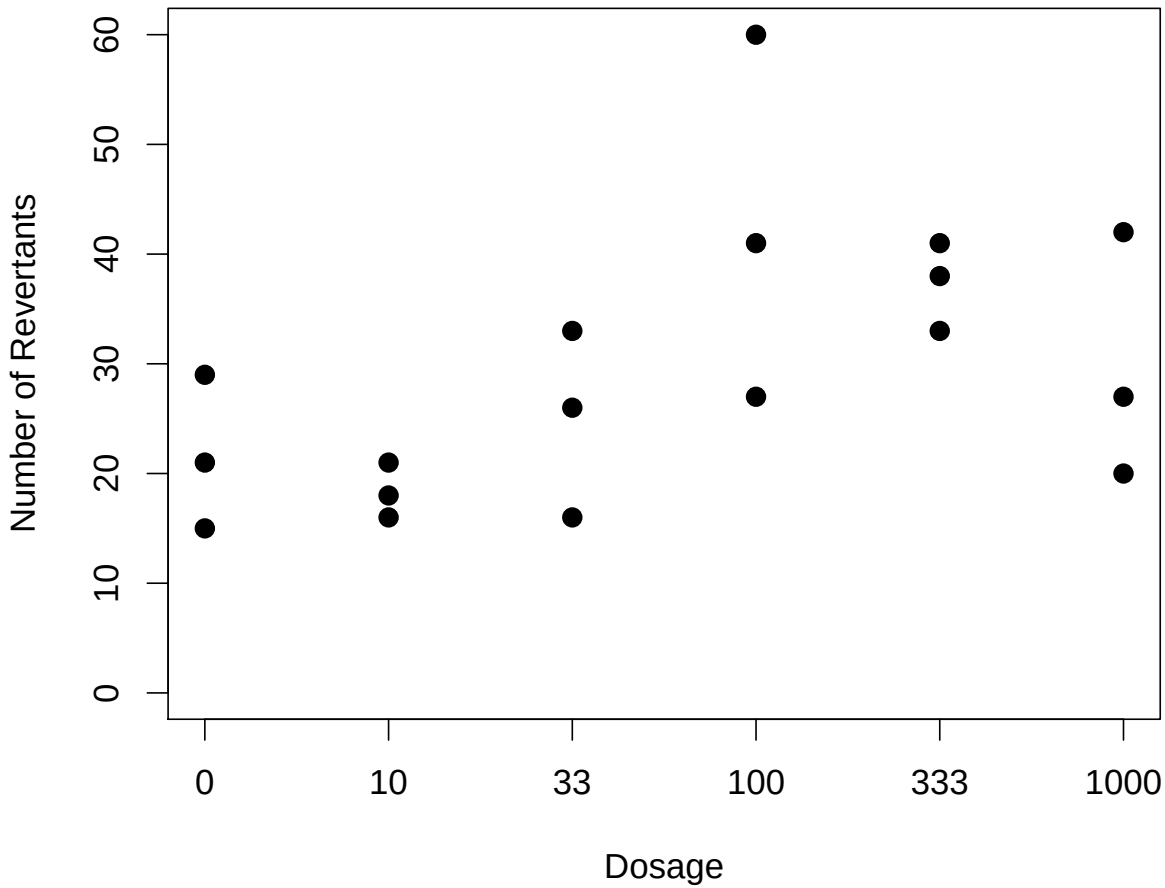
[Margolin, Kaplan, Zeiger (1981)]

- A single endpoint of revertant counts
- d=6 dosages
- r=3 independent replicates per dosage

Dose	0	10	33	100	333	1000
Revertants	15	16	16	27	33	20
	21	18	26	41	38	27
	29	21	33	60	41	42

Quinoline data (TA98, rat liver homogenate S9)

[Margolin, Kaplan, Zeiger (1981)]



Generalized Linear Model

[McCulloch and Nelder 1989]

Assuming the simple model

$$\eta_{ij} = \sum_{j}^{d} x_{ij}\beta_j, \qquad j = 1, \ldots, d;\ i = 1, \ldots, n,$$

on the log-link

$$\log(\mu_{ij}) = \eta_{ij}$$

- μ_{ij} are the predicted values for the *j*th dose and the *i*th replicate

A vector of (the logarithm of) means per dose group $\hat{\boldsymbol{\beta}}$ and it's corresponding variance covariance matrix $\widehat{\boldsymbol{\Sigma}}_{(d\times d)}$ is estimated by minimizing the Negative Binomial or Quasi-Poisson deviance.

Extra variability between plates (replicates) is considered by estimating a dispersion parameter of the Negative Binomial or Quasi-Likelihood.

Estimates for the negative binomial model

(on the log link)

Dose	Estimate	Std. Error
0	3.076	0.1595
10	2.910	0.1680
33	3.219	0.1529
100	3.753	0.1337
333	3.610	0.1378
1000	3.390	0.1459

The dispersion parameter is estimated as $\hat{\phi} = 0.03$, defined by $V(\lambda) = \lambda + \phi\lambda^2$.

Linear Functions of Model Parameters

[Bretz, Hothorn 2003]

Definition of a Williams-type contrast matrix $\boldsymbol{C} = (c_{kj})$:

	Dose					
	0	10	33	100	333	1000
C 1	-1	0.0	0.00	0.00	0.00	1.00
C 2	-1	0.0	0.00	0.00	0.50	0.50
C 3	-1	0.0	0.00	0.33	0.33	0.33
C 4	-1	0.0	0.25	0.25	0.25	0.25
C 5	-1	0.2	0.20	0.20	0.20	0.20

- Linear combinations of model parameters: $\boldsymbol{C}\hat{\beta}$
- corresponding variance-covariance matrix: $\boldsymbol{C}\hat{\boldsymbol{\Sigma}}\boldsymbol{C}'$

Approximate Simultaneous Confidence Intervals

[Hothorn, Bretz, Westfall 2008]

Lower, approximate $(1 - \alpha)$ confidence intervals for the ratio of model parameters:

$$\exp(\boldsymbol{C}\beta) \in \exp\left(\left[\boldsymbol{C}\hat{\beta} \pm z_{k,1-\alpha,\widehat{\boldsymbol{R}}}\hat{\boldsymbol{s}}\right]\right)$$

- $z_{k,1-\alpha,\widehat{\boldsymbol{R}}}$ is a quantile of the k-variate Normal distribution with correlation matrix $\widehat{\boldsymbol{R}}$
  - $\widehat{\boldsymbol{R}}$ is the correlation between the contrasts calculated by standardizing the variance-covariance matrix $\boldsymbol{C}\widehat{\boldsymbol{\Sigma}}\boldsymbol{C}'$
- $\hat{\boldsymbol{s}}$ is the square root of the diagonal elements of $\boldsymbol{C}\widehat{\boldsymbol{\Sigma}}\boldsymbol{C}'$

Results

Estimated correlation matrix:

	C1	C2	C3	C4	C5
C1	1.00	0.89	0.84	0.81	0.80
C2	0.89	1.00	0.96	0.93	0.91
C3	0.84	0.96	1.00	0.97	0.95
C4	0.81	0.93	0.97	1.00	0.98
C5	0.80	0.91	0.95	0.98	1.00

The calculated quantile of the multivariate normal distribution is 1.94

Results

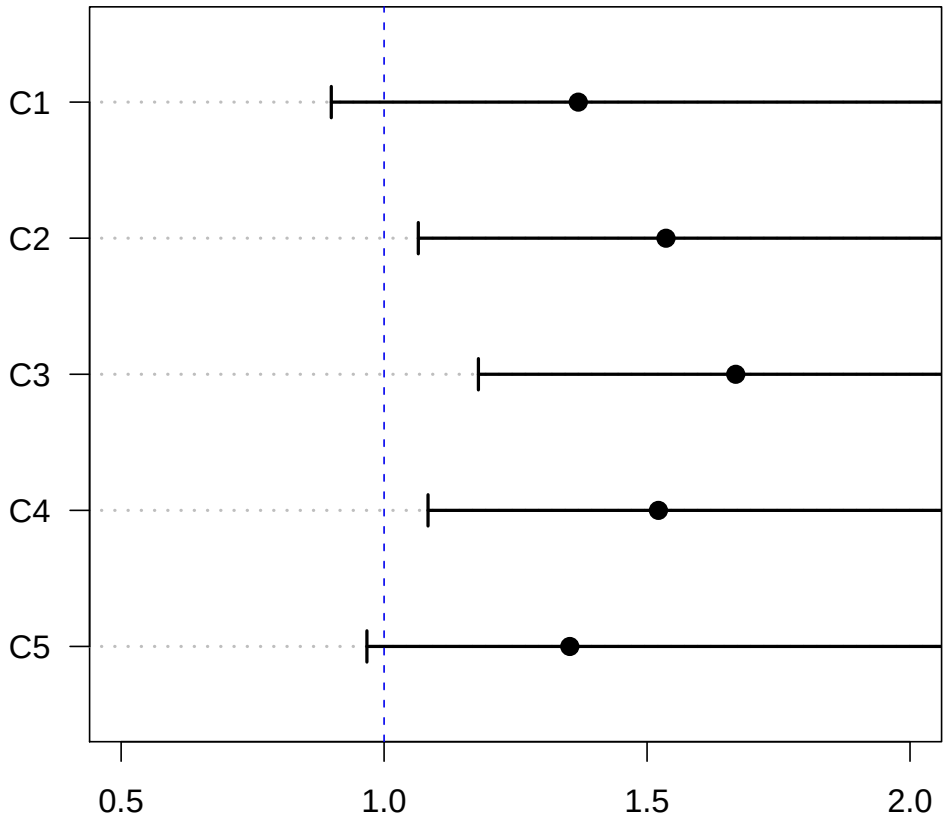
Lower, approximate $(1 - \alpha)$ confidence intervals for the ratio of model parameters:

	$\exp(\boldsymbol{C}\hat{\beta})$	lower CI
C 1	1.37	0.90
C 2	1.54	1.07
C 3	1.67	1.18
C 4	1.52	1.08
C 5	1.35	0.97

A significant trend can be observed. Maximum distance of the lower limit to 1 at contrast 3:
(-1, 0, 0, 0.33, 0.33, 0.33)

Results

Lower, approximate $(1 - \alpha)$ confidence intervals for the ratio of model parameters:



Williams Contrast with Downturn Protection

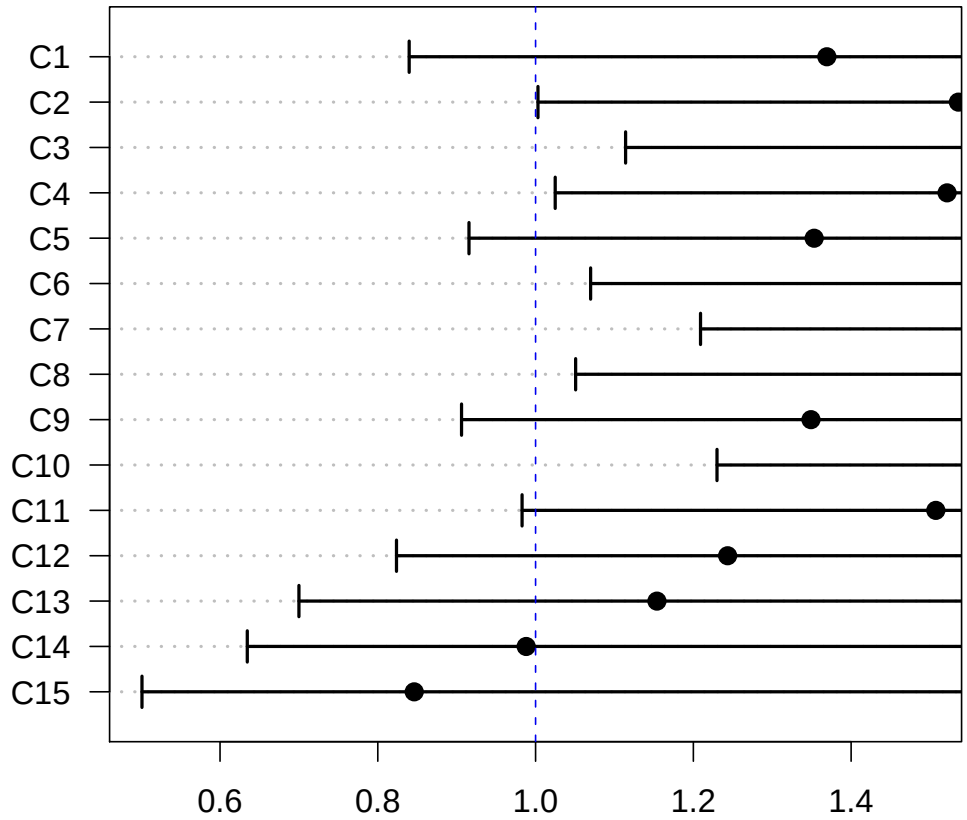
[Bretz, Hothorn 2003]

Definition of an UmbrellaWilliams-type contrast matrix $\boldsymbol{C} = (c_{ki})$:

	Dose				
	0	1	2	3	4
C 1	-1	0.0	0.00	0.00	1.00
C 2	-1	0.0	0.00	0.50	0.50
C 3	-1	0.0	0.33	0.33	0.33
C 4	-1	0.25	0.25	0.25	0.25
C 5	-1	0.00	0.00	1.00	0.00
C 6	-1	0.00	0.50	0.50	0.00
C 7	-1	0.33	0.33	0.33	0.00
C 8	-1	0.00	1.00	0.00	0.00
C 9	-1	0.50	0.50	0.00	0.00
C 10	-1	1.00	0.00	0.00	0.00

Results

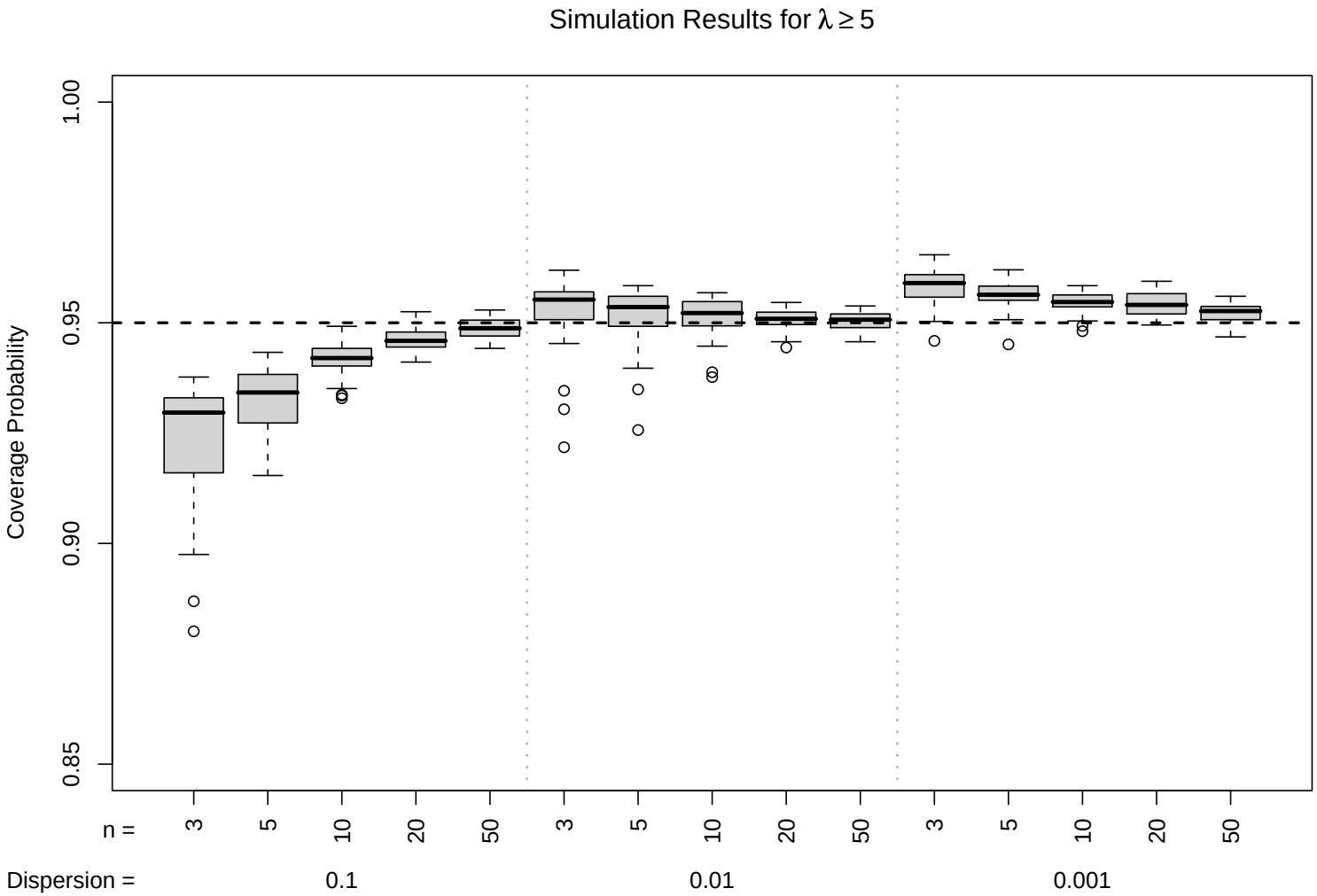
Lower, approximate $(1 - \alpha)$ confidence intervals for downturn-protected Williams-type contrasts:



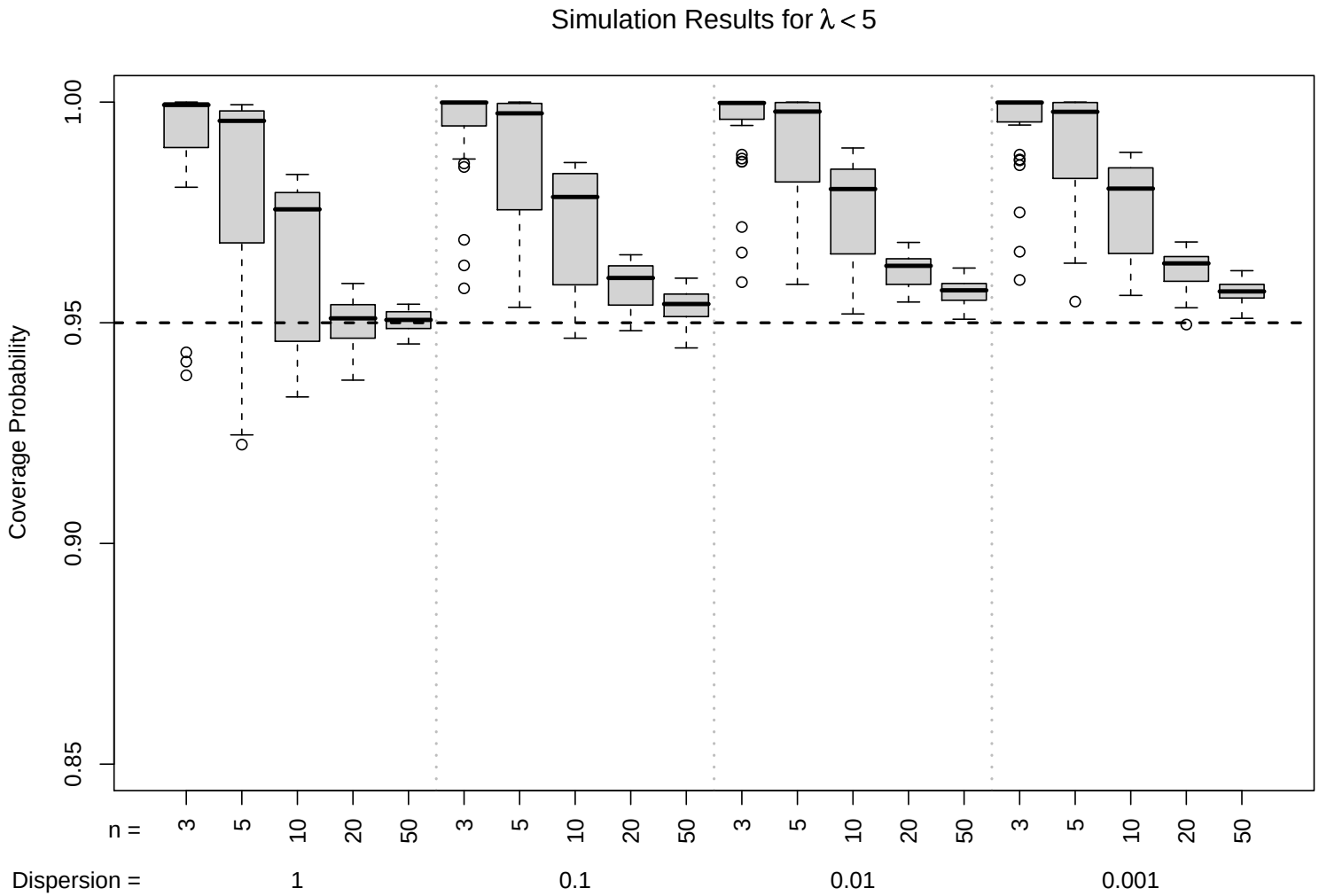
Simulation Study

- Comparison of 4 group by Williams contrasts
- Coverage probability of one-sided 0.95 confidence intervals
- Observations generated from a negative binomial distribution with
  - Mean values $\lambda = 0.5 \leq 1 \leq 2 \leq 5 \leq 10 \leq 50$
  - Dispersion parameter $\phi = 1, 0.1, 0.01, 0.001$ ($V(\lambda) = \lambda + \phi\lambda^2$)
  - Number of observations per group $n_i = 3, 5, 10, 20, 50$
- 10,000 runs per parameter combination

Coverage probability for multiple parameter combinations



Coverage probability for multiple parameter combinations



In vitro Micronucleus Assay

- Detection of micronuclei in the cytoplasm of interphase cells
- At least 3 concentrations of a test substance and a negative (and a positive) control are used
- Duplicate cultures used
- Commonly 1,000 bi-nucleated cells under observation
- Increased number of micronucleated cells corresponds to genotoxicity of the substance
- Proportion of the number of micronucleated cells to the number of scored cells is of interest

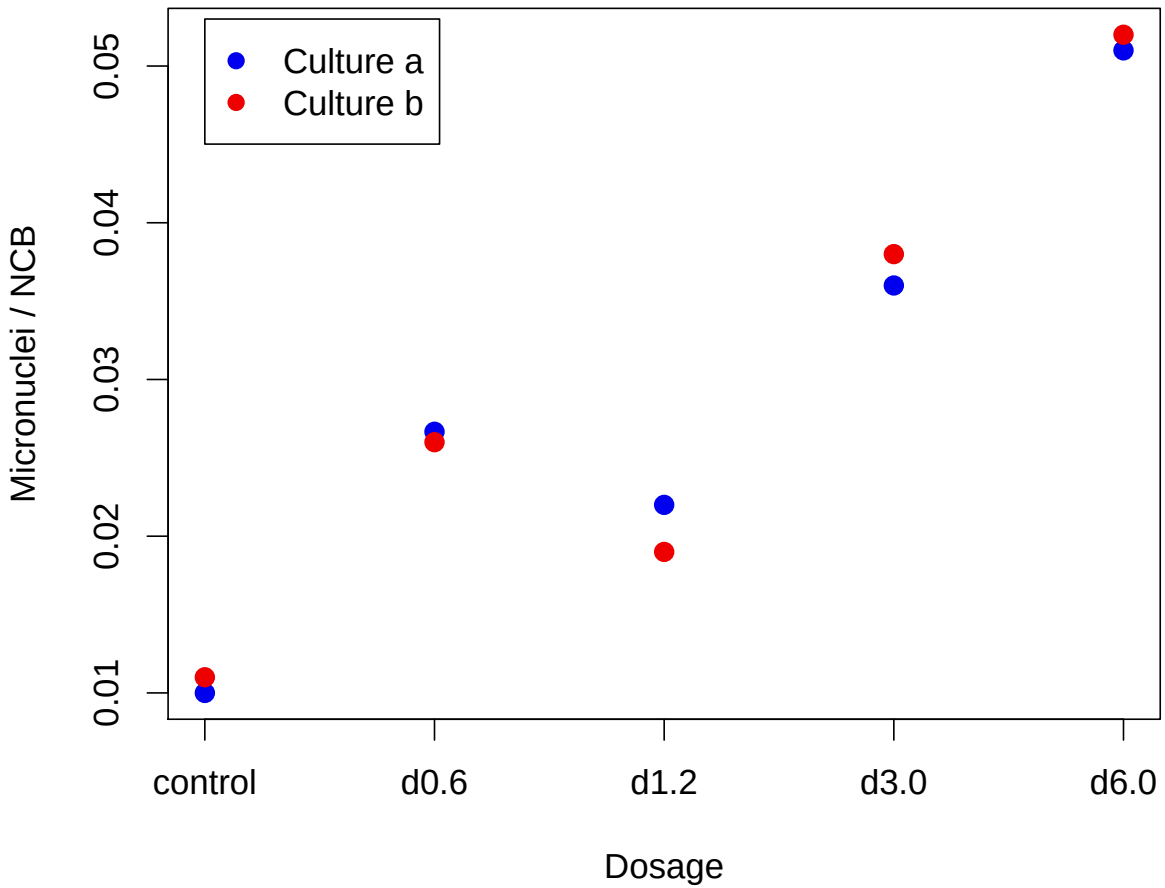
In vitro Micronucleus Assay

[Lee, Hoffman, Garriott (2003)]

Dose	Culture	NCB	Micronuclei
control	a	1000	10
control	b	1000	11
d0.6	a	600	16
d0.6	b	1000	26
d1.2	a	1000	22
d1.2	b	1000	19
d3	a	1000	36
d3	b	1000	38
d6	a	1000	51
d6	b	1000	52

In vitro Micronucleus Assay

[Lee, Hoffman, Garriott (2003)]



Generalized Linear Model

Pooling counts for cultures (a, b)
$\rightarrow$ $d \times 2$ Table:

Dose	NCB	Micronuclei
control	2000	21
d0.6	1600	42
d1.2	2000	41
d3	2000	74
d6	2000	103

- GLM assuming binomial distribution and taking a logit link
- Estimation of the log odds per dosage ($\hat{\beta}_j$)

Simultaneous Confidence Intervals

- Detection of a trend by Williams-type contrasts with contrast matrix $\boldsymbol{C}$
- Calculation of approximate lower $(1-\alpha)$ confidence intervals for the odds ratio by

$$\exp(\boldsymbol{C}\beta) \in \exp\left(\left[\boldsymbol{C}\hat{\beta} \pm z_{k,1-\alpha,\widehat{\boldsymbol{R}}}\hat{\boldsymbol{s}}\right]\right)$$

Results

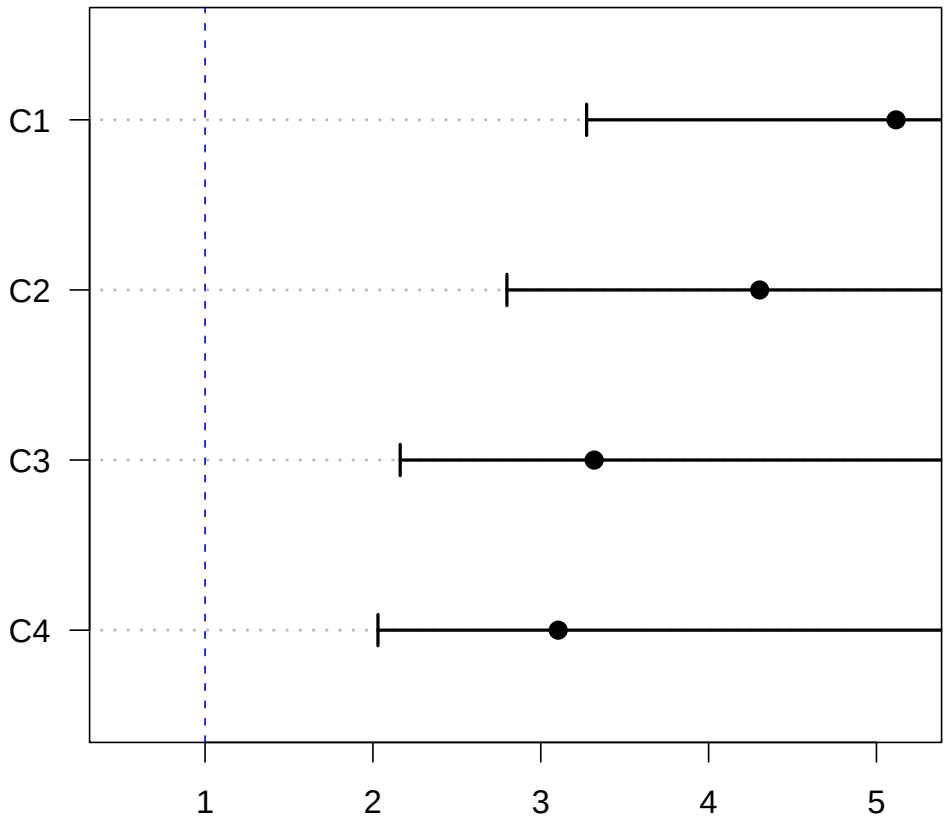
Lower, approximate $(1 - \alpha)$ confidence intervals for odds ratios, detecting a trend by Williams contrasts:

	$\exp(\boldsymbol{C}\hat{\beta})$	lower CI
C1	5.12	3.27
C2	4.30	2.80
C3	3.32	2.16
C4	3.10	2.03

A significant trend can be observed. Maximum distance of the lower limit to 1 at contrast 1:
c_{1i} = (-1, 0, 0, 0, 1)

Results

Lower, approximate $(1 - \alpha)$ confidence intervals for odds ratios, detecting a trend by Williams contrasts:



Problems

- A sample containing only zeros results in non-informative confidence intervals
- Coverage probability depended on data; problems at
  - high overdispersion
  - small proportions/means

Discussion

- Modeling counts and proportions by generalized linear models
- Multiple contrasts are unaffected by dose-response shape
- Control of the FWER by using quantiles of a multivariate normal distribution
- Confidence intervals allow reasoning for significance and biological relevance
- Free Software available

References

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