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Measuring Reproducibility of Dose Response Data for the Pig-a Assay using Covariate Benchmark Dose Analysis

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Highlights:
- Interlaboratory reproducibility is reasonable for in vivo Pig-a MF
- Covariate benchmark dose analysis provided a measure of reproducibility
- RBCs provided a greater level of Pig-a MF reproducibility and precision than RETs
- Reducing the number of zero values would increase overall reproducibility

Abstract
The reproducibility of the in vivo Pig-a gene mutation test system was assessed across 13 different Japanese laboratories. In each laboratory rats were exposed to the same dosing regimen of $N$-nitroso-$N$-ethyleurea (ENU), and red blood cells (RBCs) and reticulocytes (RETs) were collected for mutant phenotypic analysis using flow cytometry. Mutant frequency dose response data were analysed using the PROAST benchmark dose (BMD) statistical package. Laboratory was used as a covariate during the analysis to allow all dose responses to be analysed at the same time, with conserved shape parameters. This approach has recently been shown to increase the precision of the BMD analysis, as well as providing a measure of equipotency. This measure of equipotency was used here to demonstrate a reasonable level of interlaboratory reproducibility. Increased reproducibility could have been achieved by increasing the number of cells scored, as this would reduce the number of zero values within the mutant frequency data. Overall, the interlaboratory trial was successful, and these findings support the transferability of the in vivo Pig-a gene mutation assay.

Key words:
OECD
Pig-a
Benchmark dose
Potency
In vivo
Dose response
Ring trial

Introduction
During validation of the in vivo Pig-a gene mutation test system, 13 Japanese laboratories carried out a ring trial to test for assay reproducibility of the dose response following exposure to a mutagenic substance. This supports the previous international ring trial, in which the analytical techniques used to evaluate Pig-a mutation as well as the applied statistical approaches were different, but the test chemical was the same [1]. Dose responses were generated in red blood cells (RBCs) and reticulocytes (RETs) following exposure to the same set of $N$-nitroso-$N$-ethyleurea (ENU) doses. Samples were taken at weeks 0, 1, 2 and 4, with week 4 being the standard time for generating dose response data using this approach. The Pig-a assay is based on flow cytometric scoring, which allows for large numbers of events to be analysed in a quick and automated manner. Due to this ability
to provide large data sets and the increased use of mutation data for quantitative purposes
in human health risk assessment, it was considered appropriate to compare the dose
responses and to test for reproducibility. The benchmark dose (BMD) approach has recently
been championed for use in defining points of departure (PoD) for genetic toxicity endpoints
[2-5], and more recently for defining potency ranks as a measure of equipotency [6, 7].

Recent work conducted by researchers at the Dutch National Institute of Health and the
Environment (RIVM) has shown that appropriate use of BMDs in context of their confidence
limits has applications for compound potency ranking within an endpoint, as well as
empirical potency comparisons across endpoints [8-11]. Furthermore, novel computational
algorithms developed at the RIVM permit combining datasets for the same endpoint and
analogous functional form. These algorithms enable simultaneous BMD analyses to be
conducted across covariates (e.g., compound, tissue, cell type, sex, exposure
duration/regime, genotype etc.) and importantly have the potential to yield more precise
BMD estimates where normalised response shape is conserved across covariates for a
shared endpoint [12-15].

When comparing dose responses, it is essential that the data are represented on suitable
axes, and there is not any bias placed on the data through any visual critiquing. This is
achieved in the PROAST BMD analysis, through the assumption that biology is
‘multiplicative’ compared to being ‘additive’, which leads to a default log transformation of
both axes. This transformation leads to analysis of fold changes compared to absolute
changes in metrics, which are often not very comparable. Further assumptions are used
when carrying out covariate BMD analysis, including each dose response within this series of
experiments having conserved shape parameters for maximum response (c) and log-
steepness (d), while parameters for background (a), potency (b) and var (i.e., within group
variation) were covariate dependant [15]. These key assumptions are based on a recent re-
analysis of a large number of toxicological datasets indicating that the dose-responses for a
given (continuous) endpoint from different chemicals tend to have similar shapes [15]. This
approach has been tested and validated for use in potency ranking [6, 7, 15].

There are some major advantages when using the covariate approach, such as an increase in
BMD precision, because certain dose response information is used from the other dose
responses when fitting the model. Wills et al (2016b) have shown that it can be of great
benefit to include data from a study with many doses and replicates tested to improve the
BMD estimate from a study with minimal data [7]. Along with increased precision, the
discussion also moves away from whether the results are only positive or negative, to
discussions about potency. Previous efforts to measure equipotency for genotoxicity endpoints have relied on metrics such as no observed genotoxic effect levels (NOGEL) or lowest effect dose (LED), however these are imprecise estimates of potency and are highly sensitive to experimental design differences, while they do not provide a measure of uncertainty [12, 15]. The covariate BMD approach therefore provides a more suitable method for defining equipotency between different data sets, while providing further information as well.

The aim was to use the BMD covariate approach to rank the BMD metrics for Pig-a Mutant Frequency (MF) for each laboratory, to see whether the different laboratories produced BMD that were equivalent to each other.

Materials and Methods

In Vivo Pig-a assay
Table 1 provides information on Pig-a study design of the different participant laboratories, with further details in the paper within this special issue [16].

BMD Covariate Approach for Potency Ranking

Pig-a dose response datasets were obtained from the different laboratories as stated above. These data were then subjected to combined BMD analyses through combination of dose-response relationships with laboratory as covariate. Data from red blood cells (RBC) and reticulocytes (RET) were analysed separately. Pig-a mutant frequency response at 4 weeks after treatment is more stable than other earlier time points and it is appropriate to perform a covariate BMD analysis among participant laboratories. As presented in Wills et al 2016, historical dose-responses for the same endpoint but with a different chemical can be used to increase precision of the BMD estimate [7]. An extensive Pig-a MF data set containing 6 dose levels of alkylating agent methyl methanesulfonate [17] was therefore used to improve the BMD analysis in which 2 dose groups were tested for ENU. This approach allows any differences in BMDL-BMDU to be more clearly observed, by reducing the width of these BMD confidence intervals, as observed in Figures 1 and 2, which include the Zeller et al (2016) data, compared to Supplementary Figure 5 which does not.

PROAST version 61.2 was used to conduct the dose-response analyses (http://www.proast.nl). Dose-response data were analysed using both the exponential and the Hill nested model families, as recommended by the European Food Safety Authority (EFSA) for the analysis of continuous data [18]. PROAST uses the likelihood ratio test to
assess whether inclusion of additional parameters resulted in a statistically significant improvement in model fit [6, 7, 9, 12, 14]. Models with additional parameters are only accepted if the difference in log-likelihood exceeds the critical value at p<0.05 [15]. In this way, it can be established which model parameters need to be estimated for each subgroup, and which parameters may be considered as constant among the subgroups of a combined dataset. In general, it was assumed that the maximum response (parameter c) and log-steeppness (parameter d) (i.e., shape parameters) were equal for all response curves, while parameters for background response (parameter a), potency (parameter b) and var (i.e., within group variation) were covariate dependent [15]. PROAST outputs designate potency for each level of the covariate (i.e., the BMD) as CED or Critical Effect Dose, and the metrics BMDL and BMDU are designated CEDL and CEDU, respectively. Fits of the model to the datasets of each subgroup are presented in the Supplementary Figures, and were used to visually evaluate the (approximate) validity of the assumed constant shape parameters. This approach was preferred over evaluating the assumption by statistical testing, since statistical tests on the shape parameters are highly sensitive to non-random errors in the data that are ubiquitous in experimental data, and the effect of which may even be amplified by leverage effects in dose-response data [15]. Furthermore, minor non-random errors in the data might lead to rejection of the constancy of the shape parameter assumption (i.e., given the relatively high power in a combined dataset), while small differences among the shape parameters would probably only have a small impact on the coverage of the BMD confidence interval [15]. Visual inspection of the fitted curves was therefore considered a better way to determine whether any differences in parameters c and d between covariates were small enough to be ignored. Residual errors and within-group variances were visually examined for compliance to log-normality and homogeneity, respectively.

The Bench Mark Response (BMR), also known as Critical Effect Size (CES in PROAST notation), employed in the presented analyses was set at 10%. This is justified since the aim of the analyses was to examine differences in potency rather than derive a point of departure for risk assessment. The BMDL and BMDU values represent the lower and upper bounds of the two-sided 90%-confidence interval of the BMD [14], with the BMDU-BMDL ratio defining the width of the confidence interval and therefore its precision. Confidence interval plots, arranged using the geometric midpoint of the BMDL-BMDU interval were employed to visually compare potencies across levels of examined covariates whilst taking estimation uncertainty into account [19].

Results
The “maximal” (four-parameter) exponential model provided a suitable fit to the RBC and RET data at 4 weeks sampling time using PROAST (v61.2). The covariate BMD approach using constant shape parameters was used to generate Figures 1 and 2, which show the BMDL-BMDU and BMDL-BMDU plots, ranked by the midpoints of the interval [19], for RBC and RET, respectively, with the laboratory on the X axis and log_{10} of concentration (mg/kg) on the Y axis. The supplementary figures show the dose response modelling for each of these data sets, and the Hill and exponential models provide suitable fits to the data. Supplementary Figure 5 also shows the analyses carried out without data from Zeller et al., (2016), in which the BMDL-BMDU are wider and less precise which leads to more overlap between laboratories. Figs. 1 and 2 show similar results, although in three datasets (labs) the confidence intervals related to the BMD for RET were relatively large (probably due to all observations in the controls being zero, i.e. below limit of quantification, LOQ). In both figures the confidence intervals do not overlap among all labs (datasets), indicating differences among some labs. Moreover, based on visual inspection of the figures, these differences are roughly estimated to be within only one order of magnitude.

**Discussion:**

The BMD potency ranking plots provide information on the reproducibility of the Pig-a dose response data. The overlapping confidence intervals established from mutant RBC and RET frequencies at week 4 show evidence that the mutant phenotype population can be reasonably well reproduced in different datasets, i.e. within one order of magnitude. Although the differences may be due to the labs, they could also just be replication error. This could not be established here, as no replicate studies within labs were available.

This is the first instance where potency estimates using BMD covariate analysis has been used to examine interlaboratory reproducibility. Non-overlapping BMD metrics and relatively large differences between the potential values of the two BMDs would indicate that the level of reproducibility was low. In the present ring study, we found reasonable reproducibility, but it would be worthwhile to improve it. This could be achieved by increasing the number of cells scored to a minimum of 1-5x10^6 RETs or RBCs per animal, as this would reduce the number of zero’s within the data [20]. In this regard, laboratory A was the only laboratory not to contain zero data points in the control Pig-a replicates of RETs, and laboratory A also has the smallest BMDL-BMDU width. For the RBC Pig-a MF, the laboratories that did not contain any zero’s in control were B, G, L2 and O, and the BMDL-BMDU for each of these laboratories overlaps very well with each other (Figure 1).
Another example of the BMD covariate analysis approach in genetic toxicology is provided in the recent paper by Wills et al. 2016, who found no significant differences between BMD potency estimates in different experimental replicates [7]. As another example the approach was used to show the effect of sampling day in in vivo Pig-a data. The approach is robust and provides a suitable way of comparing potencies across covariates (e.g. laboratories, sampling day, compound, sex, species etc.).

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References:


Figure Legends:

Figure 1: RBC Week 4: Pig-a MF dose response data following exposure to ENU from the different laboratories was analysed using the BMD covariate approach, using BMRs of (a.) 10% and (b.) 100%. One MMS dose response data set from Zeller et al 2016 [17] was used to increase the precision of the BMD estimates [7]. The 4 parameter exponential (top horizontal lines) and Hill (bottom horizontal lines) models provided a suitable fit to the data, with 'laboratory' used as covariate. The width of the horizontal lines represents the BMDL-BMDU, which are ranked from lowest to highest concentration by BMD. During this combined analysis, the maximum response (parameter c) and log-steepness (d) parameters were assumed equal for all response curves, while parameters a (background response), b (potency) and var (within group variation) were covariate dependant. The use of constant 'shape' parameters (parameters c, d) still provided a strong description of the individual response curves. Overlapping lines show equipotency, with potency decreasing from top left to bottom right. Lab M did not produce RBC Pig-a MF. TOP: CED-0.1 is equivalent to, or another name for, BMD_{10}. BOTTOM: CED-1 = BMD_{100}. X-axes are Log10.dose(mg/kg/day); Y-axes are laboratory.
Figure 2: RET Week 4: Pig-a MF dose response data following exposure to ENU from the different laboratories was analysed using the BMD covariate approach, using BMRs of (a) 10% and (b) 100%. One MMS dose response data set from Zeller et al 2016 [17] was used to increase the precision of the BMD estimates [7]. The 4 parameter exponential (top horizontal line) and Hill (bottom horizontal line) models provided a suitable fit to the data, with ‘laboratory’ used as covariate. The width of the horizontal lines represents the BMDL-BMDU, which are ranked from lowest to highest concentration by BMD. During this combined analysis, the maximum response (parameter c) and log-steepness (d) parameters were assumed equal for all response curves, while parameters a (background response), b (potency) and var (within group variation) were covariate dependant. The use of constant ‘shape’ parameters (parameters c, d) still provided a strong description of the individual response curves. Overlapping lines show equipotency, with potency decreasing from top left to bottom right. CED-0.1 is equivalent to, or another name for, BMD$_{10}$. X-axes are Log10.dose(mg/kg/day); Y-axes are laboratory.