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Maximum Likelihood Estimation of Titer via a Power Family of Four-Parameter Logistic Model

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Abstract

For many laboratory assays, the readouts are presence or absence of a particular function, and the binary outcomes are correlated. The research interest is often focused on the estimation of titers, at which 50% positivity occurs. The classical approach by Reed and Muench (RM) assumes of a linear dose-response relationship around the potential titer, and uses only information from two points around the potential titer, which is inefficient in both precision and accuracy. While the model-based methods such as four-parameter logistic regression (4PL) use all the data, they do not consider the correlation among binary outcomes from same identities, which may lead to estimates with overstated precision. We propose estimating titers from two different anchors: independent responses from same identities or exchangeable responses from same identities. Marginal distributions of responses are linked to covariates of dilution factors by the 4PL model for independent responses and by a power family of the 4PL models for exchangeable responses. The maximum likelihood procedure is used to get estimates of parameters and titers. The superiority of proposed methods over the classical approach is demonstrated both in simulation studies and in analysis of real data from hemagglutination assays.

Keywords

Exchangeable binary data; Power family; Four-parameter logistic model

1 Introduction

Limiting dilution assay is a common tool in laboratory research, to determine the frequencies of cells having a particular function, or simply the presence of the function in cells, that are present in a mixed population of cells. All the assays start with serial dilution of test samples, with replicates at each dilution levels. In general, replicates used in assays are taken from the same test samples and thus are technical replicates instead of biological

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replicates. But in some cases both types of replicates are used. An example is hemagglutination assay.

Influenza virus particles have an envelope protein called the hemagglutinin, or HA, which binds to sialic acid receptors on cells. The virus will also bind to erythrocytes (red blood cells), causing the formation of a lattice. This property is called hemagglutination, and is the basis of the hemagglutination (HA) assay to determine levels of influenza virus present in a sample. To conduct the assay, usually two-fold serial dilutions of a virus are prepared and injected into incubated eggs. These eggs will undergo two-day incubation before being moved to cold room. Upon test some allantoic fluid will be aspirated from each egg, with several drops being put into wells with a specific amount of red blood cells. The red blood cells that are not bound by influenza virus sink to the bottom of a well and form a button. The red blood cells that are attached to virus particles form a lattice that coats the well. Here the eggs used at each dilution levels can serve as biological replicates, and the drops from each egg can serve as technical replicates. It is reasonable to assume readouts from different eggs are independent, but readouts of drops from the same egg are correlated.

For these types of assays, the research interest is often focused on the estimation of titer, which is often defined to be the 50% egg infectious dose (EID_{50})/mL (16). The question is how to analyze exchangeable binary data so that titers can be estimated. A simple but classical way is using linear interpolation by Reed and Muench (15), assuming a linear dose-response relationship around the potential titer. But the Reed-Muench method uses only information from two points around the potential titer, and thus it is inefficient in both precision and accuracy. Another way is to model the dose-response curve. There is a large literature on using mathematical models to fit bioassay data, such as a probit model (3), a logistic regression model and Gomperts distribution (SAS, 2002), four-parameter logistic models (18). These models can be used in the scenario we are describing, but the correlation between readouts from the same identities (such as eggs in the HA assay) have to be considered for improved precision. Responses from same identities are more likely to be same than responses from different identities. Ignoring the intra-identity correlation may lead to estimates with overstated precision.

As pointed by Bowman and George (4) and Kuk (9), in many studies, various correlated binary data obtained from the same litter, dam, individual or cluster are exchangeable, in the sense that, a sequence of random variables $Y_1, Y_2, \dots, Y_n$ is exchangeable if for any n and any vector $(y_1, \dots, y_n)$,

$$P(Y_{\pi(1)} = y_1, \dots, Y_{\pi(n)} = y_n) = P(Y_1 = y_1, \dots, Y_n = y_n), \quad (1)$$

for any permutation $\pi(1), \dots, \pi(n)$ of $1, \dots, n$. We borrow the idea, and assume that the binary readouts from the same identity (eggs in the HA assay) are exchangeable with the property (1).

In summary, we propose to model the correlated binary data by dose-response curve with exchangeable covariance structure. In Section 2, we review the theories behind exchangeable

binary data. In the next section, we present the models depicting the dose-response curve under the exchangeable covariance structure model, and the corresponding inference. In Section 4, we conduct extensive simulation studies to explore the possible consequences of ignoring the exchangeable property inherent in the data, and illustrate its application in analysis of real data from the HA assay. The paper is concluded with some discussion in Section 5.

2 Statistical Analysis of Exchangeable Binary Data

Let $Y_{ij1}, \dots, Y_{ijn_{ij}}$ be n_{ij} exchangeable binary random variables from the j th identity at the i th dilution level, for g dilution levels with $i=1, \dots, g$ and m_j identities at each dilution levels with $j=1, \dots, m_j$.

The work by Bowman and George (4) and by Kuk (9) demonstrated that, if $Y_{ij1} = \dots = Y_{ijn_{ij}} = 1$ and $Y_{ij(r_{ij}+1)} = \dots = Y_{ijn_{ij}} = 0$, the joint probability of $Y_{ij1}, \dots, Y_{ijn_{ij}}$ is

$$P\left(Y_{ij1}, \dots, Y_{ijr_{ij}}, Y_{ij(r_{ij}+1)}, \dots, Y_{ijn_{ij}}\right) = \sum_{l=0}^{n_{ij}-r_{ij}} (-1)^l \binom{n_{ij}-r_{ij}}{l} \lambda_{i, r_{ij}+l}, \quad (2)$$

with

$$\lambda_{i,k} = P(Y_{ij1} = 1, Y_{ij2} = 1, \dots, Y_{ijk} = 1) \quad (3)$$

for $k=1, \dots, n_{ij}$ and $\lambda_{i,0} = 1$. Let $R_{ij} = \sum_{l=1}^{n_{ij}} Y_{ijl}$ and $r_{ij} = \sum_{l=1}^{n_{ij}} Y_{ijl}$. Thus

$$P(R_{ij} = r_{ij}) = \binom{n_{ij}}{r_{ij}} \sum_{l=0}^{n_{ij}-r_{ij}} (-1)^l \binom{n_{ij}-r_{ij}}{l} \lambda_{i, r_{ij}+l}. \quad (4)$$

Denote the correlation of order k at the dilution factor of i by $\rho_{i,k}$, then

$$\left(\text{Var}(Y_{ij1})\right)^{k/2} \rho_{i,k} = E(Y_{ij1} - \mu_i) \dots (Y_{ijk} - \mu_i),$$

where $E(Y_{ij1}) = \lambda_{i,1} = \mu_i$. Thus

$$\rho_{i,k} = \frac{\sum_{l=0}^k (-1)^{k-1} \binom{k}{l} \lambda_{i,1}^{k-1} \lambda_{i,l}}{\{\lambda_{i,1}(1 - \lambda_{i,1})\}^{k/2}} \quad (5)$$

The log-likelihood function can be calculated as

$$l(\beta) = \sum_{i=1}^g \sum_{j=1}^{m_i} \log P(R_{ij} = r_{ij})$$

$$= \sum_{i=1}^g \sum_{j=1}^{m_i} \log \left\{ \binom{n_{ij}}{r_{ij}} \sum_{l=0}^{n_{ij}-r_{ij}} (-1)^l \binom{n_{ij}-r_{ij}}{l} \lambda_{i,r_{ij}+l}(\beta) \right\} \quad (6)$$

3 Modeling Dose-Response Curve under Exchangeable Covariance Structure

How to choose the link function F which links $\lambda_{i,k}$ to X_i ? A sufficient condition on F is that F is a completely monotone function, in other words, $(-1)^k F^{(q)}(k, \beta) \geq 0$ for all positive integers q . In the past, following folded logistic function (4) was used

$$\lambda_k = \frac{2}{1 + (k+1)^{\beta_0 + \beta_1 x}}. \quad (7)$$

There are several limitations associated with the folded logistic model. First, there are no additional parameters to model the correlation structure. The intra-identity correlation is fixed automatically once the response probability is given, which is unrealistic and restrictive. In addition, the model $\gamma_0 + \gamma_1 x$ is not enough to depict the dose-response curve in immunoassays, which gives maximum value for non-diluted samples and minimum value for infinite dilutions, and is approximately linear in the middle-dose range.

In response to the inflexibility of the folded logistic model, Kuk (9) proposed the power family of completely monotone functions which allows flexible modeling of any dose-response curve. We borrow the idea as below,

$$\lambda_{ik} = P(Y_{ij1} = \dots = Y_{ijk} = 1) = p_i^{k^\gamma}, \quad (8)$$

where $0 < p_i = \lambda_i$ is the response probability at dilution level i , and $\gamma \leq 1$. Here γ is used to measure intra-identity correlation with $\gamma = 1$ corresponding to independence and $\gamma = 0$ corresponding to complete dependence. In this way, the intra-identity correlation will not only depend on response probabilities p_i and thus can be flexibly modelled.

3.1 Dose-response Curve

A well-studied dose-response model for binary assay data is the four-parameter logistic regression model (4PL)

$$p_i = f(x_i, \beta) = \frac{\beta_3 - \beta_2}{1 + (x_i/\beta_4)^{\beta_1}} + \beta_2, \quad (9)$$

where $p_i = \lambda_i$, the dilution level x_i is usually in $\log_2$ or $\log_{10}$ scale depending on serial dilution factor (2-fold or 10-fold), and the parameters are biologically meaningful, with β_3 representing the maximum response, β_2 being the minimum response, β_4 being concentration that results in 50% response, and β_1 denoting the relative slope around 50% response (β_4). The 4-parameter logistic regression model has been successfully applied to radioimmunoassays, ELISA, pneumococcal opsonophagocytic killing assay and others (17; 6; 2; 1; 19; 7; 18). For simplicity, we will use this commonly used model for illustration, but other similar models such as the five-parameter logistic model (14) can be used as well.

Thus our power family of dose-response is

$$F(x_i, \beta) = \lambda_{ik} = \left(\frac{\beta_3 - \beta_2}{1 + (x_i/\beta_4)^{\beta_1}} + \beta_2 \right)^{k^\gamma} \quad (10)$$

and the corresponding log-likelihood function for a set of exchangeable binary data from specific immunoassays is

$$l(\beta) = \sum_{i=1}^g \sum_{j=1}^{m_i} \log \left\{ \binom{n_{ij}}{r_{ij}} \sum_{l=0}^{n_{ij}-r_{ij}} (-1)^l \binom{n_{ij}-r_{ij}}{l} \left(\frac{\beta_3 - \beta_2}{1 + (x_i/\beta_4)^{\beta_1}} + \beta_2 \right)^{(r_{ij}+l)^\gamma} \right\} \quad (11)$$

3.2 Optimization Algorithm

The commonly used optimization methods such as the Newton-Raphson and the quasi-Newton methods rely entirely on derivatives of functions to be maximized. These methods work well when the objective function is regular and smooth over the parameters' domain, but otherwise they often fail (5). Even with smooth objective functions, the sparsity of real data may bring difficulties to optimization methods relying on derivatives. On the other hand, a genetic optimization algorithm (GOA) (13) combines the quasi-Newton method with an evolutionary algorithm, which uses a set of heuristic rules to generate trial solutions in the direction of giving better fitness values compared with its predecessor. This global optimization algorithm does not require the objective function to be smooth or continuous. Thus the GOA was used to maximize the log-likelihood. Despite its powerful function, it is hazardous to rely on the GOA's default settings. Although our objective function is a smooth function of parameters, the dichotomy of the responses and the usually small sample sizes

from immunoassays add great difficulties to optimization. Our extensive numerical study by both simulations and real data analysis suggests the determination of parameters' domains is also important for the algorithm to promptly converge to a reasonable point, since the algorithm starts with a randomly generated starting value from the selected domain. The GOA may stop at a point which is biologically meaningless with a domain which is too wide, or stop at a false maximizer with a domain which is too narrow. Fortunately all of the parameters in our proposed model are biologically meaningful, and their ranges come in default. This gives us great advantage in applying the GOA.

3.3 Titer Estimation & Inference

Once model fitting and parameter estimation is accomplished by the GOA, the outcome of interest such as titers x_{50} shall be obtained by inversion of the fitted curve. In the general case, let $h(y, \beta)$ be the inverse function of the monotonic mean function $f(x, \beta)$. Then a natural estimator of x_{50} is $\hat{x}_{50} = h(50, \hat{\beta})$, where $\hat{\beta}$ is the parameter estimator of β with asymptotic covariance matrix $V(\beta, \gamma)$. The parameter covariance matrix $V(\beta, \gamma)$ is typically estimated by the inverse of the expected information matrix $-H^{-1}(\hat{\beta}, \hat{\gamma})$, or the inverse of the negative of the observed information matrix $-H^{-1}(\hat{\beta}, \hat{\gamma})$ (aka Hessian matrix). In the case of 4-parameter logistic regression model with or without intra-identity correlation, we have

$$\hat{x}_{50} = \exp\left(\frac{1}{\hat{\beta}_1} \log\left(\frac{\hat{\beta}_3 - 50}{50 - \hat{\beta}_2}\right) + \log \hat{\beta}_4\right). \quad (12)$$

The variance estimation of $\hat{x}_{50}$ can be obtained by the bootstrap procedure (19) or processed by the Delta method as,

$$\widehat{Var}(\hat{x}_{50}) = h'_{\beta}(50, \hat{\beta}) V(\hat{\beta}, \hat{\gamma}) h_{\beta}(50, \hat{\beta}), \quad (13)$$

where $h_{\beta}(y, \beta)$ represents the derivative of h with respect to β , $(\hat{\beta}, \hat{\gamma})$ are MLE estimators for (β, γ)

4. Numerical Study

4.1 Monte Carlo Simulations

Monte Carlo simulations were applied to validate the superiority of the power family of dose-response models over the RM method, and also the common modeling approach using 4PL ignoring the intra-identity correlation. That is, how the newly proposed models work better when the binary data under study is truly exchangeable, even with additional parameter to be estimated under typical small sample sizes for immunoassays. We used R package of *binarySimCLF* to simulate correlated binary data with exchangeable covariance structure. Specifically, the 4PL was used as the dose-response curve, with $\beta = (5, 0.001, 0.991, 3)$. The choice of β_3 and β_2 was fully based on their biological representations, and β_1 and β_4 were based on real data analysis. Thus the true x_{50} was set at 2.99 using formula

(12). To gauge the effect of intra-identity correlation parameter γ on titer estimation, we selected γ to be 0.1, 0.5 and 0.9, across the full valid range of this parameter. We used the power function (10) to get $\lambda_{i,k}$, and the correlation function (5) to get intra-identity correlations. With marginal response probabilities and intra-identity correlations, *binarySimCLF* was used to generate sets of exchangeable binary outcome.

For simplicity, we assumed a balanced design, that is, number of identities were **equal to** m across dilution levels and number of replicates from each identity was **equal to** n across identities and dilution levels. The assay is straightforward, involving very few design factors. To mimic experimental scenarios and also to evaluate the performance of the power function of 4PL in a broad range, we let $m = 2, 3$, $n = 2, 3, 4$, and the total number of dilution levels $g = 8$.

To assess the relative performance, we studied the empirical density curve of each estimator, and compared their absolute relative errors (ARE) and sum of squared error (SSE). ARE is defined as

$$ARE_b = \frac{|\hat{x}_{50_b} - x_{50}|}{x_{50}} \quad (14)$$

where b denotes the b th simulation run, with $b = 1, \dots, 1000$. The reported ARE was the average of AREs from 1000 simulation runs.

SSE was calculated from

$$SSE = \sum_{b=1}^{1000} \left(\hat{x}_{50_b} - x_{50} \right)^2 \quad (15)$$

Figure 1 presents the empirical density functions of titer estimators by the exchangeable 4PL, independent 4PL and RM methods. The density curves for the RM estimators are consistently more flat and **have several peaks**, suggesting titer estimators by this approach are not stable but across a wider range. To the contrary, the estimators by the exchangeable 4PL and independent 4PL are more centralized around the true titer, though the exchangeable 4PL estimators have higher peak at the true titer. Table 1 presents the ARE and SSE for all three estimators under small, medium and large intra-identity correlation. As indicated, the exchangeable 4PL performs very stable in terms of both ARE and SSE despite the strength of intra-identity correlation, while the independent 4PL comes next. Overall, both of the model-based estimators are much more accurate than the RM estimators according to both criteria.

4.2 Real Data Examples

We used three methods to estimate titers in a data set from the hemagglutination assay (10; 12). The study was designed to explore how primary influenza viral replication **affects**

cellular immune responses. To measure viral concentrations, lung homogenates underwent 6 serial two-fold dilutions, and were then inoculated into 3 embryonated hen eggs at each dilution level before 2 replicates were taken from each egg to determine the 50% egg infective dose (EID₅₀)/ml. Table 2 includes the raw assay data for two participants. We applied the three methods to the data, and presented means, standard deviations, minimum and maximum of titer estimators for two participants, to compare consistency and variability of titer estimation among three methods. Bootstrap procedure with 500 resampling was used to estimate the standard deviations.

Overall, the exchangeable 4PL and the independent 4PL give close estimators, while the RM estimators depend on the smoothness of dose-response curve. The dose-viral presence proportion curves for both participants were approximately linear in the middle dilution range, which explains smaller variability of the RM estimators. For both participants, the intra-egg correlations were estimated as 0.1, suggesting relatively strong intra-egg correlations.

5. Discussion

Hemagglutination assay is a classical approach to estimate viral titer. The traditional model-based methods using 4PL or 5PL could be, and often **were**, used in such assays to estimate neutralizing antibody titers. However, they do not consider the correlation among binary outcomes from same identities, which may lead to estimates with overstated precision. We assumed the correlated binary outcomes are exchangeable, and modeled the mean curve by 4PL under the exchangeable framework. Although the 5-parameter logistic regression model or others could be used as well, **our purpose was not** on the model development, but combining the typical dose-response curve modeling with exchangeable covariance structure. In addition, the sparsity of real data brings numerical difficulty to 5-parameter logistic models. The simulation study **indicated** that the exchangeable 4PL performs better than the independent 4PL, and both model-based approaches outperform the RM method regardless of sample size and intra-identify correlation. Traditional optimization algorithms work well with smooth objective functions of non-sparse data which is uncommon in reality. The global optimization algorithm (13) combines the quasi-Newton method with an evolutionary algorithm, and thus overcomes the non-smoothness and sparsity in real data. The exchangeable 4PL is indeed more computationally intensive, requiring about 1 minute compared to average 20 second for the independent 4PL for each bootstrap run of the real data. But this computational load is completely acceptable in practice. The proposed exchangeable 4PL can be used in similar assays producing correlated binary outcomes for other diseases. Statistical software R was used for data analysis and simulation studies.

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Figure 1:
Empirical Density Curves of Estimators. ex.est, Titer estimator by Exchangeable 4PL; ix.est, Titer estimator by Independent 4PL; rm.est, Titer estimator by Reed-Muench method

Table 1:

Ex, Exchangeable 4PL; Ix, Independent 4PL; RM, Reed-Muench method. ARE, average relative error; SSE, sum of squared error.

$\gamma =$		0.1						0.5						0.9					
$m =$		2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4
$n =$		2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4
ARE	Ex	0.137	0.136	0.133	0.117	0.117	0.121	0.126	0.118	0.113	0.102	0.098	0.094	0.110	0.091	0.083	0.090	0.081	0.067
	Ix	0.145	0.148	0.146	0.120	0.122	0.125	0.133	0.122	0.121	0.107	0.104	0.099	0.116	0.094	0.086	0.092	0.083	0.068
	RM	0.183	0.199	0.206	0.193	0.197	0.201	0.188	0.194	0.201	0.175	0.155	0.169	0.178	0.158	0.148	0.155	0.125	0.118
SSE	Ex	248	236	224	164	167	179	202	176	162	135	125	118	158	105	96	108	89	62
	Ix	299	301	304	199	204	215	251	209	206	157	147	138	189	118	104	119	96	65
	RM	544	632	688	550	600	592	522	557	602	446	368	412	470	327	306	336	217	185

Table 2:

Raw assay data for two participants.

		Dilution Factor								
		1	2	3	4	5	6			
ID78	1	1	1	1	0	1	1	0	0	0
	2	1	1	1	1	1	1	0	0	0
	3	1	1	1	1	0	0	0	0	0
ID98	Eggs	5	6	7	8	9	10			
	1	1	1	0	0	0	0	0	0	0
	2	1	1	1	1	0	0	0	0	0
	3	1	1	1	1	1	1	0	0	0

Table 3:

Ex, Exchangeable 4PL; Ix, Independent 4PL; RM, Reed-Muench method.

		Mean	S.D.	Min	Max
	Ex	4.176	0.740	2.434	4.907
ID78	Ix	4.171	0.739	2.426	4.900
	RM	3.992	0.370	2.667	4.500
	Ex	7.352	0.492	6.180	8.606
ID98	Ix	7.345	0.486	6.181	8.567
	RM	7.489	0.383	6.500	8.400