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CALIBRATION AND USE OF AN IN-HOUSE ANTI-MEASLES IgG STANDARD SERUM

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SUMMARY

For the purpose of research a large quantity of anti-measles IgG working reference serum was needed. A pool of sera from five teenagers was prepared and named Alexandre Herculano (AH). In order to calibrate the AH serum, 18 EIA assays were performed testing in parallel AH and the 2nd International Standard 1990, Anti-Measles Antibody, 66/202 (IS) in a range of dilutions (from 1/50 to 1/25600). A method which compared parallel lines resulting from the graphic representation of the results of laboratory tests was used to estimate the power of AH relative to IS. A computer programme written by one of the authors was used to analyze the data and make potency estimates. Another method of analysis was used, comparing logistic curves relating serum concentrations with optical density by EIA. For that purpose an existing computer programme (WRANL) was used. The potency of AH relative to IS, by either method, was estimated to be 2.4. As IS has 5000 milli international units (mIU) of anti-measles IgG per millilitre (ml), we concluded that AH has 12000 mIU/ml.

KEYWORDS: Calibration; Measles; IgG; Reference serum.

INTRODUCTION

Some commercial kits for enzyme immunoassays (EIA) provide standards to permit the transformation of optical density values in categories of "immune", "non-immune" and "borderline or equivocal", or similar designations. This approach to measure anti-measles IgG (mIgG) has a limited precision (no concentration value is provided) and may be misleading because mIgG concentration in the standards provided is not known.

The need to standardize serological assays performed in different laboratories to measure anti-measles IgG has been recognized[4,5,11]. To assist in the standardization, the Expanded Programme on Immunization (EPI) recommends the use of the International Standard for anti-measles serum[8] which contains 5000 milli international units per millilitre, after reconstitution. Because of the importance and limited quantity of an international standard, it has been recommended that primary standards should be used to calibrate a working standard prepared in house[11]. This serum can then be used to convert to international units, the results of the assays measuring serum anti-measles IgG (mIgG) in study sera.

For the purposes of a study, we needed to measure mIgG in a large number of sera. Here, we describe the calibration process of a working standard and how it was subsequently used in the assays to measure mIgG.

MATERIALS AND METHODS

We used a commercial enzyme immunoassay (EIA) (14458 Measles Virus IgG EIA, Diagnostica, Merck) to measure measles IgG in human sera.

Calibration of an anti-measles IgG working standard serum

In February 1994, a measles epidemic occurred in a secondary school, Alexandre Herculano (AH), in Oporto. Five students aged 15-17 years, who described a clinical condition compatible with measles, gave blood samples after informed consent from their parents and themselves. Blood was collected 5-7 weeks after the disease. A preliminary assay, using IS as the standard, showed that all five sera had measles IgG concentrations well above 5000 mIU/ml. A pool of sera from the five teenagers was then prepared and named AH, after the school.

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The design and analysis of the calibration assays were based on general recommendations for this type of study[7,8,13]. To calibrate the AH serum, 18 dilution series[10,14,15] were performed, testing AH and the IS in parallel (Tables 1 and 2). Three dilution series per plate, and one plate per day, were tested on six separate occasions, on different days. Both sera were used in the same dilutions. In each of series 1 to 6, sera were diluted 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600, and in each of series 7 to 18, sera were diluted 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400. An initial sample dilution of 1:50 was set up, with 20 µl of serum and 980 µl of dilution buffer. From that one, successive doubling dilutions were prepared. New tips were used in each pipetting step. The dilution series from AH and IS were tested in contiguous positions, using the left, central and right areas of the plate, alternating the order of the two sera in a balanced design as recommended in the literature[8]: the sequence IS, AH, AH, IS, IS, AH in one plate was alternate in the following plate with the sequence AH, IS, IS, AH, AH, IS.

Data analysis

In the analysis, series 11 was rejected because technical mistakes made it impossible to know exactly the dilutions and places used, and series 18 was rejected because, due to a malfunction of the automatic washer, the last column of the plate could not be washed in standard conditions.

From each series, plots were generated of the optical density reading against reciprocal dilution. As an example, Figure 1 shows optical density (OD) values obtained for IS and AH in series 14.

Two methods were used to analyze the data; one based on parallel line assays, while the other fitted a logistic curve:

"Parallel line assays". The principles and methods applicable to parallel line assays[1,7,13], were used. First, 18 plots were produced with the results of the series comparing AH with IS (Tables 1 and 2). Optical density (OD) values (untransformed) were plotted against the reciprocal of the dilutions (a log scale). After observing the plot for each series, points were chosen or rejected, in order to fit two parallel lines corresponding to each of the sera. In most cases the rejected points were the extreme high and low concentrations. After that, the method of analysis of parallel line assays was used to estimate the potency of AH relative to IS. A computer programme, written in Basic by one of us (Maia JC) for the purpose of this study, was used. The mathematical basis for this analysis has been described in the literature[5,6,7] and is not detailed here, but the general steps were:

a) a straight line was fitted to the data from each serum using the least squares regression method;

b) the probability of the two regression lines being parallel was determined; series in which the probability of parallelism was below 0.05 were rejected from further analysis (only series 10 was rejected on this basis);

c) the estimate of the potency of one serum relative to the other was based on the calculation of the horizontal distance between the two parallel lines (eg Fig. 1);

d) the final estimate of potency, combining the results from different assays, was computed taking the geometric mean of the individual potency estimates[13].

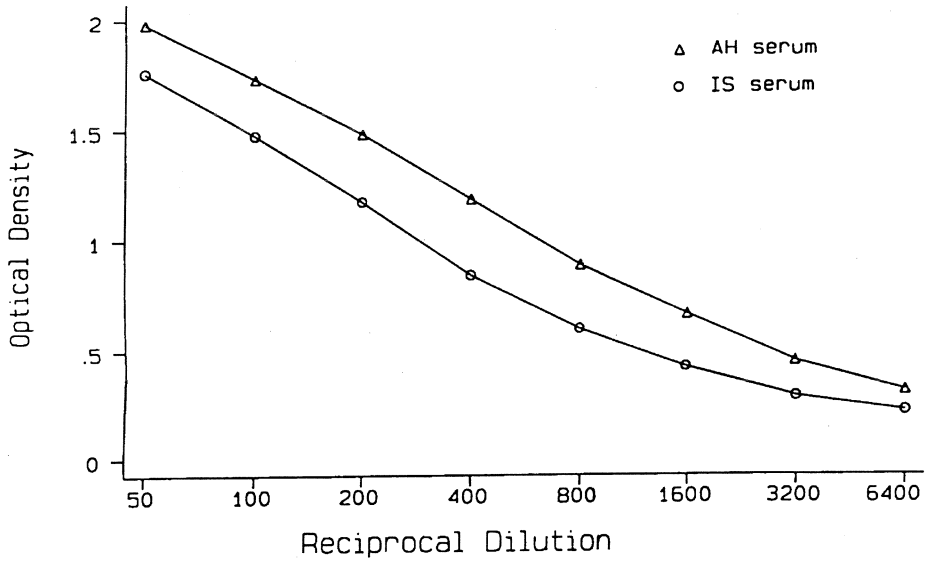

Fig. 1 - Calibration of AH serum against the International Standard (IS). Optical density responses by reciprocal of dilutions of serum. Series 14.

TABLE 1
Results from the calibration assays (series 1 to 9). Absorbance values by EIA

Reciprocal serum dilution	Series 1		Series 2		Series 3	
	IS	AH	IS	AH	IS	AH
50	—	—	—	—	—	—
100	1.439	1.937	1.541	2.157	1.589	1.976
200	1.226	1.487	1.282	1.677	1.300	1.655
400	0.870	1.223	0.955	1.415	0.985	1.345
800	0.655	0.951	0.815	1.069	0.832	1.086
1600	0.471	0.723	0.484	0.808	0.591	0.905
3200	0.320	0.476	0.466	0.513	0.422	0.560
6400	0.212	0.352	0.315	0.400	0.242	0.477
12800	0.252	0.203	0.264	0.304	0.384	0.281
25600	0.120	0.275	0.259	0.314	0.153	0.333
Reciprocal serum dilution	**Series 4**		**Series 5**		**Series 6**	
	IS	AH	IS	AH	IS	AH
50	—	—	—	—	—	—
100	1.470	1.870	1.527	1.930	1.445	1.852
200	1.143	1.628	1.192	1.592	1.156	1.536
400	0.865	1.317	0.916	1.272	0.966	1.377
800	0.616	0.978	0.580	0.961	0.707	1.070
1600	0.427	0.685	0.399	0.649	0.491	0.793
3200	0.278	0.456	0.281	0.442	0.342	0.509
6400	0.192	0.299	0.203	0.291	0.229	0.354
12800	0.143	0.205	0.136	0.209	0.184	0.206
25600	0.145	0.147	0.127	0.144	0.130	0.170
Reciprocal serum dilution	**Series 7**		**Series 8**		**Series 9**	
	IS	AH	IS	AH	IS	AH
50	1.682	2.031	1.672	1.970	1.926	2.213
100	1.406	1.738	1.365	1.739	1.598	1.999
200	1.086	1.467	1.106	1.399	1.265	1.661
400	0.788	1.133	0.929	1.232	0.919	1.299
800	0.559	0.836	0.630	0.907	0.626	0.960
1600	0.368	0.570	0.427	0.591	0.444	0.666
3200	0.252	0.384	0.284	0.398	0.296	0.436
6400	0.161	0.283	0.194	0.255	0.214	0.284
12800	—	—	—	—	—	—
25600	—	—	—	—	—	—

Legend: IS=International Standard; AH=serum to be calibrated
Note: Sera were not placed in the plates in the order seen in this tables. They were placed in the left and right extremes of the plates and in the middle using the scheme: IS/AH/AH/IS/IS/AH, AH/IS/IS/AH/AH/IS etc.

TABLE 2
Results from the calibration assays (series 10 to 18). Absorbance values by ELISA

Reciprocal serum dilution	Series 10		Series 11		Series 12	
	IS	AH	IS	AH	IS	AH
50	1.542	2.115	—	—	1.720	2.067
100	1.298	1.742	—	—	1.467	1.789
200	1.077	1.536	—	—	1.204	1.585
400	0.783	1.174	—	—	0.925	1.339
800	0.560	0.987	—	—	0.616	0.999
1600	0.366	0.638	—	—	0.473	0.707
3200	0.251	0.397	—	—	0.337	0.535
6400	0.177	0.276	—	—	0.271	0.349
Reciprocal serum dilution	**Series 13**		**Series 14**		**Series 15**	
	IS	AH	IS	AH	IS	AH
50	1.653	1.996	1.758	1.980	1.766	2.164
100	1.364	1.698	1.474	1.730	1.486	1.793
200	1.056	1.425	1.171	1.479	1.191	1.560
400	0.806	1.230	0.837	1.181	0.898	1.253
800	0.551	0.887	0.593	0.880	0.628	0.957
1600	0.332	0.580	0.418	0.658	0.436	0.674
3200	0.248	0.392	0.279	0.443	0.277	0.447
6400	0.164	0.259	0.213	0.307	0.215	0.332
Reciprocal serum dilution	**Series 16**		**Series 17**		**Series 18**	
	IS	AH	IS	AH	IS	AH
50	1.628	2.073	1.714	2.060	—	—
100	1.415	1.851	1.457	1.805	—	—
200	1.128	1.552	1.113	1.536	—	—
400	0.815	1.252	0.832	1.228	—	—
800	0.564	0.943	0.560	0.882	—	—
1600	0.439	0.667	0.395	0.617	—	—
3200	0.331	0.490	0.271	0.432	—	—
6400	0.194	0.273	0.205	0.293	—	—

Legend: IS=International Standard; AH=serum to be calibrated
Note: Sera were not placed in the plates in the order seen in this tables. They were placed in the left and right extremes of the plates and in the middle using the scheme: IS/AH/AH/IS/IS/AH, AH/IS/IS/AH/AH/IS etc.

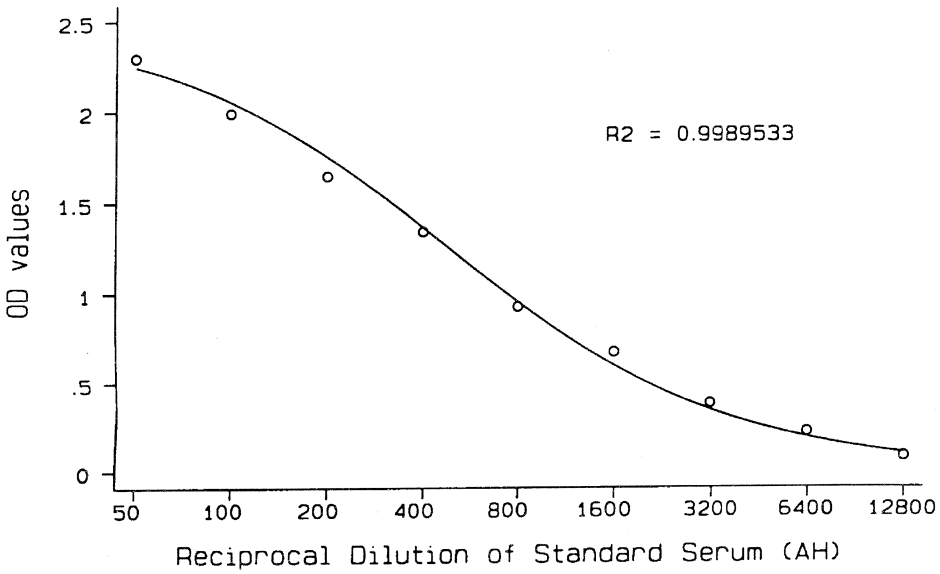

Fig. 2 - Logistic curve fitted to the optical density values (OD) by dilution of the standard serum (AH). Determination of measles IgG levels by EIA. Plate 31.

Logistic curve analysis. As seen in Figure 1, the general shape of the plots of OD against reciprocal dilution is closely sigmoid. Thus, a logistic function fitted the observed data very well, as reported for most bioassays[6].

Two of us (Heath A and Walker D) used the comparison of logistic curves to estimate the potency of AH related to IS, using a previously made computer programme (WRANL programme)[8]. This programme has an inbuilt procedure that weights the analysis to the central parts of the curves, which tend to be the areas that are used for the parallel line analysis. The rationale for this is the assumption that the area of the curve with the steepest slope should have the most information for determining differences between potencies of the test and the standard sera, while data close to the upper and lower limits (where both curves tend to converge) would be less reliable for discriminating between the two. Series 10 was excluded to allow direct comparison with the parallel line method. The procedure used was as follows:

a) Estimates of the upper limit of the curves (one for each series) were obtained from a non-linear curve fitting procedure, using the SAS statistical package, for each curve separately;

b) for each assay, a single estimate for the upper limit was taken as the maximum of the estimates for the individual curves;

c) the data were transformed to percentages of the upper limit and the WRANL program was used to determine relative potencies[6].

We also tried fitting a lower limit to the curves (instead of assuming a lower limit of zero), and we tried taking means of the estimates of the upper limit, rather than the maximum. The method above was selected on the basis of plots of the data after linearising transform had been applied.

Conversion of OD values to mIU/ml of anti-measles IgG in the study sera

Serum AH was subsequently used in a study of passive immunity against measles, in which about 3000 sera were studied (maternal and cord sera)[9]. In each plate, the study sera were diluted 1/200. The AH standard serum was used in the dilutions of 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400 and 1/12800. The optical density (OD) response values were used to draw a curve relating the logarithm of the dilution (the log of concentration in mIU can also be used) with OD. As observed during the calibration process, the figures produced showed that the general shape of the lines through the plotted points was closely sigmoid. A Basic computer programme written by one of us (Maia JC) tested the fitting of a logistic curve to the observed OD values by reciprocal dilution (Fig. 2) (the same could be done with the concentration fitted as natural logs).

The programme also derived the parameters of the logistic curve with the best fit. Those parameters (specific for each of the forty plates) were used in an Epi-Info programme which converted values of OD into mIU/ml for all study sera tested.

When an OD value of a study serum was above the OD value corresponding to AH serum diluted 1/50 in the same plate, a new determination was made using a 1/800 dilution of the study serum. Conversely, if an OD value of a study serum was below the OD value corresponding to AH serum diluted 1/12800, a new determination was made using a 1/50 dilution of the study serum.

In the first two plates used in the calibration process, we also used the standards provided by the manufacturer to classify sera as "positive" or "negative". The programme mentioned above was used for each of the first 6 series, used in the calibration process, of both the International and the AH serum, in order to estimate the concentration of mIgG corresponding to the cut-off proposed by the manufacturer's method.

TABLE 3
Estimates of potency of Alexandre Herculano (AH) serum relative to the International Standard for anti-measles serum (IS) (GMP = geometric mean potency)

Series No.	POTENCY	
	Method 1 (parallel line model)	Method 2 (WRANL)
1	2.196	2.285
2	2.431	2.573
3	2.460	2.400
4	2.664	2.682
5	2.319	2.367
6	2.910	2.693
7	2.247	2.263
8	2.302	2.144
9	2.222	2.196
10	—	—
11	—	—
12	2.521	2.570
13	2.405	2.448
14	2.070	2.094
15	2.278	2.270
16	2.674	2.781
17	2.363	2.430
18	—	—
GMP	2.395	2.405
95%CI	2.295, 2.501	2.292, 2.523

RESULTS

Calibration of an anti-measles IgG working standard serum

Table 3 shows the potency estimates from each series and the final point estimate of potency (geometric mean and 95% CIs) of AH against IS, by each method. The two methods gave very similar results. The geometric mean potency point estimates were very close to 2.4, with similar and narrow confidence intervals. Thus, for the purposes of subsequent mIgG measurements, the potency of AH relative to IS was set as 2.4. As IS has 5000 milli international units (mIU) of anti-measles IgG per millilitre (ml), AH was estimated to have 12000 mIU/ml.

TABLE 4
Estimates of the concentration of anti-measles IgG (in mIU/ml) corresponding to the cut-off proposed by the manufacturer of the enzyme immunoassay kit. Values were estimated fitting a logistic function to each standard serum curve and series.

Series	Standard Serum	
	Internatinal	Alexandre Herculano
1	350	394
2	222	280
3	236	245
4	333	334
5	343	353
6	245	279

Conversion of OD values to mIU/ml of anti-measles IgG in the study sera

The fitting was always excellent with R^2 values close to 0.999 for all forty plates that were used (minimum of 0.99857 for one of the plates). As an example, Figure 2 shows the curve derived from Plate 31.

Using the optical density (OD) readings for the standards and the formula given by the manufacturer, the cut-off OD values to classify sera as "positive" or "negative", would have been 0.494 in the first plate (dilution series 1 to 3) and 0.439 in the second plate (dilution series 4 to 6). Using IS and AH dilution series to estimate the corresponding values in mIU/ml (Table 4), we can see that the cut-off correspond to values ranging from 222 mIU/ml to 394 mIU/ml (Table 4), well above the concentration value of 200 mIU/ml, considered to be the threshold of immunity[3].

DISCUSSION

Calibration of an anti-measles IgG working standard serum

The two methods gave similar results and the confidence intervals were reassuringly narrow. The similarity of the results from the two methods results from the inbuilt weighting in the WRANL programme, that effectively weights the analysis to the central parts of the curves. These tend to be the areas that are used for the parallel line analysis without transformation. Intuitively, the part of the curve with the steepest slope should have the most "information" for comparing potencies between the two preparations, data close to the upper and lower limits being less reliable for discriminating between the preparations.

Method 2 uses all the information but is more complex to perform. The parallel assay method (Method 1) has the potential weakness of relying on subjective criteria at some point of the procedure, and rejecting the information from the upper and lower

levels. Nonetheless, parallel line methods are easier to understand than the more complex method 2, and it is remarkable that the simpler, sound and well established Method 1 gave a very close result to Method 2, in which a carefully constructed and already tested computer programme and corresponding procedure were used.

Conversion of OD values to mIU/ml of anti-measles IgG in the study sera

The calibration process followed by the use of several dilutions of the working standard in each plate, described here, was an elaborate procedure, but it was useful to allow quantification of EIA results[11]. Developing a standard curve in EIA tests permits the detection of smaller differences than any method that depends on serial dilution[2]. In a previous study on passive immunity, measles IgG was measured in maternal, cord and infants sera, using EIA and a reference curve[12] so that the results were expressed in mIU/ml. In that study, the serum used for the reference curve was the IS[8] but it was not clear how many and which dilutions of the standard serum were used. Because square roots of concentration were used in that study, the curve fitted to OD values by square root of mIU/ml was not a sigmoid but a parabola. As in our study, the fitting was excellent, with an R^2 value of 0.99[12].

The main issue in the calibration process is the choice of dilutions and above all the standard serum to be used. Since the supply of the International Reference Serum is limited, laboratories should calibrate their own standard sera for use in large studies. For EIA kits in general, some manufacturers provide calibrated standards and instructions to build reference curves, either using programmable machines, or even more approximate methods like the use of graphic extrapolations from plots of values for the standards. Sometimes, as in this case, the manufacturers of measles IgG kits, do not provide precisely calibrated standards, but kits come with semi-quantitative standards to permit the transformation of the OD values in categories of "positive" and "negative". We showed that the optical density cut-off value would correspond to a concentration value above 200 mIU/ml, resulting in false negative results.

Whenever recognized international standards exist, they should be preferred to commercial standards[11] though there may be a need to calibrate in-house standards for large studies. We recommend that, whenever the manufacturer provides a standard serum, it should be verified whether that was calibrated against the IS, and if so, how it was done. This applies to EIA kits measuring antibodies other than measles IgG.

Needless to say, compliance with the manufacturer's recommendations when performing EIA tests is paramount, including checking if the OD values of the standard controls provided are valid. A preliminary observation of a plot of the standard curve is fundamental. That gives the observer an idea about the best model/s fitting the curve and can show if something wrong has happened. Thus, it should prevent the effect of a "black box" when relying on automatic procedures.

RESUMO

Calibração e uso de um soro padrão de referência de IgG anti-sarampo

Necessitávamos de uma grande quantidade de soro padrão para ser usado num estudo em que seria doseada IgG anti-sarampo em centenas de soros, usando uma técnica imunoenzimática (EIA). Foi preparado um homogeneizado de soros de cinco adolescentes ao qual foi dado o nome de Alexandre Herculano (AH). Para calibrar o soro AH foram realizados 18 ensaios pela técnica EIA, avaliando em paralelo AH e o soro padrão internacional (SPI)(2nd International Standard 1990, Anti-Measles Antibody, 66/202) em diluições sucessivas de 1/50 a 1/25600. Para estimar a potência do soro AH relativamente ao SPI, foi usado um método no qual se compararam linhas paralelas representando graficamente os resultados da prova laboratorial. Para a análise dos dados e estimativa da potência, foi usado um programa informático escrito para o efeito por um dos autores (Maia JC). Foi usado outro método de análise, no qual foram comparadas curvas logísticas representando a relação entre concentrações séricas de IgG anti-sarampo e os correspondentes valores de densidade óptica (DO) pela EIA. Para este efeito foi usado um programa informático já conhecido (WRANL) por dois de nós (Heath A e Walker D). A potência estimada do soro AH relativamente ao SPI foi de 2,4. Como o SPI tem uma concentração de IgG anti-sarampo de 5000 mili unidades internacionais por mililitro (mUI/ml), concluimos que o soro AH tem 12000 mUI/ml.

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