COLLABORATIVE STUDY FOR THE ESTABLISHMENT OF THREE PRODUCT SPECIFIC EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATIONS FOR INACTIVATED ADSORBED HEPATITIS A VACCINES

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1. INTRODUCTION

Hepatitis A vaccines prepared by formalin inactivation of purified cell-culture grown hepatitis A virus are licensed in several European countries. The potencies of these vaccines are expressed in units unique to each manufacturer. Standardisation of potency statements requires establishment of an international reference material. At its 1999 meeting, the ECBS[2] established a preparation coded 95/500 as the 1st IS for hepatitis A vaccine, inactivated.

When the WHO study was being planned, the EDQM proposed an international collaborative study to assess candidate reference materials for use as Ph. Eur. BRPs to assist the batch release of hepatitis A vaccines by OMCLs. As with previous projects, EDQM and WHO agreed that, to avoid duplication of effort, the two studies should be combined. This report describes the part of the study to evaluate the BRPs. Inactivated hepatitis A vaccines can be tested in antigen content and immunogenicity assays. The candidate reference materials were tested in both types of assay to provide information on the correlation between the antigen content and immunogenicity of each preparation. This study was performed under the aegis of the Biological Standardisation Programme and supported by the Council of Europe, the European Commission and WHO.

2. AIM OF THE STUDY

The aims of the study were:

a) to determine if any of the candidate EDQM reference materials are suitable as Ph. Eur. BRPs for inactivated hepatitis A vaccine;

b) to calibrate the proposed Ph. Eur. BRPs against the IS;

c) to investigate the correlation between antigen content and immunogenicity assays of the candidate reference materials;

d) to determine if a common standard antigen content method is suitable for the assay of various inactivated hepatitis A vaccines.

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(2) Abbreviations: BRP: Biological Reference Preparation; ECBS: WHO Expert Committee on Biological Standardisation; EDQM: European Directorate for the Quality of Medicines; EIA: Enzyme-Immuno-Assay; GCV: Geometric Coefficient of Variance; IS: International Standard; HAV: Hepatitis A Vaccine; NIBSC: National Institute for Biological Standards and Control; OD: Optical Density; OMCLs: Official Medicines Control Laboratories; PBS: Phosphate Buffered Solution; Ph. Eur: European Pharmacopoeia (Pharmacopée Européenne); QC: Quality Control; RIA: Radio-Immuno-Assay; SOP: Standard Operating Procedure; WHO: World Health Organisation.

3. PARTICIPANTS

A total of sixteen laboratories from eleven countries, including both manufacturers and OMCLs, participated in the study, each of which is referred to in this report by an arbitrarily assigned number, not representing the order of listing at the end of the report.

4. MATERIALS AND METHODS

4.1. MATERIALS

Ph. Eur. BRP candidate reference materials

Four candidate materials from four manufacturers were used; each is representative of vaccines on the market. They were coded:

E, F, G: Three formalin inactivated alum adjuvanted antigens from three manufacturers. Each is formulated to the manufacturer's licensed human vaccine specification. These preparations are offered as aqueous candidate reference materials.

H: A formalin inactivated antigen from another manufacturer that is adjuvanted in combination with influenza proteins and formulated in an identical manner to licensed human dose vaccine. This preparation is offered as an aqueous candidate reference material.

Additional study materials

The following materials were also provided to the participants:

A: A formalin inactivated antigen formulated in an identical manner to a licensed human vaccine except for the omission of the usual alum adjuvant. This antigen is not freeze-dried and long-term storage is at – 70 °C. The 1999 WHO ECBS established this material as the 1st IS for hepatitis A vaccine, inactivated.

B: An antigen (sample A) exposed to an environmental stress known to cause loss of potency.

C: A coded duplicate of the 1st IS (sample A).

D: A freeze-dried formalin inactivated antigen shown to be immunogenic in humans in clinical trials. Included in the study as a possible alternative to the 1st IS.

4.2. METHODS

Participants were requested to test the study samples in antigen content and immunogenicity assays. Participants were also asked to perform in common a standard antigen content assay proposed by the EDQM, in parallel with their in-house antigen content assay (if available). The candidate standard antigen test is a modification of a commercially available kit for anti-HAV antibodies and details of the method were supplied with the test samples and are appended to this report (Appendix 1). Where a laboratory performed more than one assay method, each method was treated as though it had been performed by a different laboratory.

Study design

Immunogenicity assays

Participants were asked to test the eight study samples. It is usual to test samples concurrently in a collaborative study. However, concurrent immunogenicity tests of all eight samples were not feasible. Participants were therefore asked to adhere to a testing program that resulted in three independent assays of the 1st IS (sample A) and the possible alternative WHO standard

(sample D), two of the candidate EDQM reagents and one of the other study materials. This was to be accomplished over four assays with four preparations per assay. The scheme to be used was given in the protocol.

Participants were requested to perform an assay that produces a dose response, for example, the Ph. Eur. mouse immunogenicity assay, or any other suitable method. Suggested dilution ranges were supplied with the samples, but a preliminary assay of one sample was recommended to determine if the suggested dilution series was appropriate in each laboratory. If not, then laboratories had discretion to scale their dilutions appropriately. Details of methods used are given in Table 8. Raw assay data were to be returned to NIBSC for analysis.

Antigen content assays

Participants were asked to test the same eight antigens as studied by the immunogenicity assays. They were requested to assay each preparation three times for antigen content using a candidate standard method and three times with their in-house method, if available. Each assay was to include all samples (unless otherwise specified, see below) and separate assays were to be carried out at least one week apart. A standard plate layout was to be used for the candidate standard method and, if possible, for the in-house method as well.

A standard plate layout was given in the protocol that required six dilutions per sample and at least two dilution series per sample. Each assay consisted of two 8×12 plates. To avoid possible edge effects, the samples were to be concentrated towards the centre of the plate and the longer edges were to be reserved for blanks, or other controls. The shorter edges could be used for repeating a sample dilution series. This was thought to be preferable to not using these wells at all and would give additional information on possible edge effects. The positions of the samples on the plates were varied across assays, but it was important for the laboratories to vary the time of application to the plate in line with the sample layouts (rather than put on, for example, sample A, first, whatever its position). Participants were asked to return the exact layout and order of application that was used.

Suggested dilutions for each preparation were supplied with the samples. However, participants were encouraged to carry out a preliminary assay to determine if the suggested dilutions were appropriate and to modify their dilutions if necessary.

Because three of the candidate Ph. Eur. BRPs are alum adjuvanted, desorption of antigen was required prior to the antigen assay. Preliminary experiments have not identified a common desorption process. Therefore product specific desorption methods had to be used, details of which were supplied with the samples. However, as manufacturers do not wish their methods disclosed to competitors, the desorption method was only made available to OMCLs in countries where a particular manufacturer's product is licensed. Therefore, not all laboratories studied samples E, F and G.

Raw assay data were to be returned to NIBSC for analysis. Participants were also requested to return their own potency estimates of samples B, C, D, E, F, G and H using sample A, the 1st IS, as the standard.

Statistical analysis

Immunogenicity assays

All assays were analysed by fitting parallel line probit models for quantal response bioassays (Finney 1978). The proportion of units responding plotted against log dose will give a sigmoid (S-shaped) dose response curve. The sigmoid curve is linearised by replacing each response, that is the proportion of units responding, by the corresponding value of the cumulative standard normal distribution. The deviations from linearity and parallelism are approximately χ^2 distributed, leading to the usual statistical tests of validity.

Antigen content assays

Each plate was treated as a separate assay. All assays were analysed as parallel line bioassays (Finney 1978), comparing assay response with log concentration. When plotted against log concentration, linear response (or transformed response) lines which are parallel for all preparations included in the assay, are essential for this analysis. The statistical validity of linearity and parallelism of the assays was assessed by analysis of variance.

Individual responses or pairs of responses that contributed significantly to heterogeneity of variance between groups of responses within an assay were omitted. Less than 0.5 % of the responses were omitted because of contributions to heterogeneity. Responses at the extremes of the response range that showed no change with further increase or decrease of dose were omitted. In some cases, the linear part of the curves were chosen or doses of some preparations were omitted to restrict analysis to areas where the response range was common to all preparations included in the assay, to achieve linearity and parallelism.

For over half of the laboratories a log transformation of the responses was found to give best linearity with log dose. However, for the majority of the remaining laboratories, the dose-response curve was sigmoid in shape. Data were transformed to percentages relative to the upper and lower limits of dose-response lines for each assay. An in-house program (WRANL, Gaines Das and Tydeman 1980) was used to provide weighted regression analysis of logit response on log dose with an assessment of linearity and parallelism, and estimates of relative potency. For one laboratory, the reciprocal of the responses was found to be more satisfactory.

Potency assignments of samples

For both the immunogenicity and the antigen content assays, the potencies of samples B, C, D, E, F, G and H relative to sample A, the 1st IS, were calculated for each assay. Similarly, the potencies of the Ph. Eur. BRP candidate samples were recalculated against each of the other Ph. Eur. BRP candidates and the results compared. For each laboratory, combined potency estimates were obtained by taking geometric means of results from all assays and overall potency estimates were calculated as geometric means of the laboratory means. Variability within laboratories (between assays) and between laboratories was measured by calculating % GCV's (Kirkwood 1979).

5. RESULTS

5.1. ASSAY DATA

The sixteen participants contributed data from a total of 125 assays, excluding any preliminary assays to determine optimum dilution ranges - 48 immunogenicity assays and 77 antigen content assays involving 142 plates. Eleven laboratories performed immunogenicity assays, thirteen performed the candidate standard antigen content assays and ten laboratories performed in-house antigen content assays. Deviations from the study protocol and other anomalies were as follows:

(i) Laboratory 02 returned raw data from antigen content assays by the candidate standard method and an in-house method which both used the recommended plate layout. However, they also performed in-house antigen content assays that did not follow the recommended plate layout, but did not return the raw data from these assays. Therefore, NIBSC estimates of potency could not be calculated for this method and the laboratories' own estimates were used instead. Their immunogenicity assays did not follow the recommended layout either. Instead, two assays were performed. The first one included the WHO samples only and the second the Ph. Eur. BRP samples only. Therefore, the estimated potencies of the Ph. Eur. BRP samples, relative to the WHO samples, could not be calculated.

(ii) Laboratory 04 did not have enough samples to perform all of the antigen content assays for samples E, F, G and H. In addition, they did not follow the recommended plate layout, but assayed the WHO samples on the first plate and the Ph. Eur. BRP samples on the second plate of each assay. Therefore, the estimated potencies of the Ph. Eur. BRP samples, relative to the WHO samples, could not be calculated.

(iii) Laboratories 08, 12 and 17 could only measure samples A, B, C, D and H as they had no product-specific method to desorb samples E, F and G. Laboratory 02 did not assay sample B in their in-house antigen content assays and laboratory 07 did not assay sample H on the second plate of their third candidate standard antigen content assay.

(iv) Laboratory 08 measured all samples of one assay on one single plate. In addition to the three assays requested, a fourth candidate standard antigen content assay was performed with 1 % Zwittergent in the dilution buffer (laboratory 08B), which is a prerequisite for testing virosomal vaccines. Laboratory 01 also commented that in their experience, the antigen content of virosomal vaccines determined without Zwittergent pre-treatment is considerably lower than with Zwittergent pre-treatment.

(v) Laboratory 09 returned data from two additional immunogenicity assays which included repeats of sample H with sample A and samples F or G. In their additional assays (laboratory 09B), sample H was diluted in PBS for injection into mice.

(vi) In the first immunogenicity assay of laboratory 13, sample D failed to seroconvert any of the animals whereas good responses were obtained in the third and fourth assays. An investigation did not reveal whether or not there was a problem with the dosing of the mice or with the EIA evaluation of the sera. There was insufficient sample or sera to retest. Therefore, sample D was excluded from this assay.

(vii) The in-house antigen content assays of laboratory 13 did not follow the recommended plate layout. The first assay involved three plates and the other two assays two plates each. However, each sample was only assayed on one plate within an assay. Therefore, not all relative potencies could be calculated.

(viii) Laboratory 15 had to repeat some of their tests because they reported "the kit which they used showed really 'individual' character for the OD value of the test sera, for example, the negative test sera OD values were in the range of 0.396-1.96".

(ix) Sample B was not tested in the immunogenicity assays of laboratory 17A since it was shown to be negative in the immunogenicity assays of laboratory 17B. In addition, they reported that the results from the first immunogenicity assays of laboratories 17A and 17B did not correlate for an unidentified reason.

5.2. Assay validity for immunogenicity assays

Sample D was omitted from the first immunogenicity assays of laboratories 15 and 17A, due to non-parallelism and the fact that the estimated potency of sample D, relative to sample A, by the first immunogenicity assay of laboratory 15 was approximately ten times that of the fourth assay. Because of the failure of sample D to seroconvert any of the animals in the first immunogenicity assay of laboratory 13, this assay was omitted when estimates of potency of the samples were recalculated relative to sample D. Tests of validity were performed at 5 % significance level. The assumptions of linearity and parallelism of the log dose-transformed response lines of samples A, B, C, D, E, F, G and H held separately in 98 % and 96 % of the immunogenicity assays, respectively. Both linearity and parallelism held in 94 %. All immunogenicity assays were included in subsequent analysis.

5.3. Assay validity for antigen content assays

Laboratory 04 omitted several responses from the extreme doses of various samples in a number of antigen content assays because they were out of the range of their standard curve.

Similarly, laboratory 13 excluded several interpolated/extrapolated responses from the extreme doses of various samples in a number of their candidate standard antigen content assays. In addition, some of the highest doses in the antigen content assays of laboratories 10, 11 and 13, both the candidate standard and the in-house assays, resulted in responses too high for their machine to read and were excluded from subsequent analysis. Laboratory 10 reported a lack of parallelism of sample B in their in-house antigen content assays. Therefore, their results should be treated with caution. The estimated potency of sample D, relative to sample A, obtained in the second in-house antigen content assay by laboratory 13 was approximately four times higher than that obtained by their other assays. Because of this, and the fact that laboratory 13 did not determine the potency estimate of sample D in this assay, sample D was excluded from this assay in subsequent analysis.

Tests of validity were again performed at 5 % significance level. The assumptions of linearity and parallelism of the log dose-transformed response lines of samples A, B, C, D, E, F, G and H held separately in 85 % and 37 % of all antigen content assays, respectively. Both linearity and parallelism held in 35 %.

Slopes of response lines were compared across all antigen content assays. Samples B and H were found to be significantly non-parallel to all other samples, but not to each other. If slopes of response lines are compared across the candidate standard assays only, then the same conclusion is reached. However, if slopes of response lines are compared across the in-house assays only, then sample H is found to be significantly non-parallel from samples A and D, but no other significant non-parallelism is present. These results are reflected in the number of valid assays if samples B and H are in turn omitted, as shown in Table 1.

Table 1 — *Validity of antigen content assays*

	Antigen content (CS & IH)			**Antigen content (CS)**			**Antigen content (IH)**		
	% linear	% parallel	% valid	% linear	% parallel	% valid	% linear	% parallel	% valid
All preparations included	85	37	35	84	31	30	87	46	42
Edges with sample B or H omitted	84	38	35	82	30	29	87	49	45
Sample B only, omitted	85	46	43	83	38	37	88	57	51
Sample H only, omitted	85	49	45	85	40	38	86	64	57
Samples B and H omitted	85	67	61	83	61	55	88	77	70

CS: candidate standard method; IH: in-house method.

It is not surprising that problems with parallelism exist for samples B and H since these samples are of a much lower potency than the other samples. Sample B was pre-treated in a way that was expected to cause loss of potency. After the study was in progress, information was provided which suggested that 1 % Zwittergent should have been included in the diluent for assays of sample H. Therefore sample H was not tested with an optimised assay and antigen content results for this sample may be unusually low.

Omitting edges of the plate which contained samples B or H did not increase the number of valid assays considerably (Table 1). If both samples B and H are omitted, then the assumptions of linearity and parallelism of the log dose-transformed response lines of samples A, C, D, E, F and G hold separately in 85 % and 67 % of all antigen content assays, respectively. Both linearity and parallelism hold in 61 %. The corresponding percentages for the candidate standard assays are slightly lower and slightly higher for the in-house assays (Table 1).

However, some of the invalidities may have been due to inadequate replication in the assay design, as well as from real deviations from the model assumptions. Therefore, all antigen content assays and all samples, except samples B and H, were used in subsequent analysis. To obtain potency estimates of samples B and H from the antigen content assays, for comparison with those from the immunogenicity assays, analysis was based on the antigen content assays omitting sample H and the antigen content assays omitting sample B, respectively. However, these results should be treated with caution because of the high proportion of invalid assays noted above and in Table 1. There was no consistent evidence of any edge effects. Therefore, responses obtained from the edges of plates were included in subsequent analysis.

5.4. POTENCIES OF SAMPLES USING THE 1ST IS AS THE STANDARD

Laboratory mean potency estimates of samples C, D, E, F, G, B and H relative to sample A, the 1st IS, assuming sample A has a potency of 1.0, by the immunogenicity assays and antigen content assays (both candidate standard and in-house methods) are listed in Tables 2a-2c, along with 95 % confidence limits for the NIBSC calculations, and the laboratories' own calculations of potency. The data are also shown in histogram form (for the NIBSC calculations) in Figures 1a-1g. It should be noted that where sample A is absent from a plate or an assay, then the estimates of potency were calculated relative to sample C, the coded duplicate of sample A. Estimates of potency of sample C are only shown if both samples A and C were included in the same plate or assay.

The histograms (Figures 1a-1g) illustrate good agreement between laboratories and assay methods for samples C, D, E, F, G and B. Agreement is not as good for sample H. For some of the samples, the immunogenicity assays give a wider spread of results than the antigen content assays. The in-house antigen content assays give a slightly wider spread of results than the candidate standard antigen content assays, for sample D, and possibly samples G and B. The overall spread of the results is highest for sample H. In addition, the immunogenicity assays appear to give higher potency estimates of sample H, and possibly samples E and F, than the antigen content assays. However, the reverse is possibly true for samples G and B. As noted above the antigen content assays for sample H may not have been optimal. The effect of adding 1 % Zwittergent to the dilution buffer is shown in the differences between the results for lab 08A (no Zwittergent) and lab 08B (with Zwittergent) in Table 2c. Antigen content results in this report for sample H must be treated with caution.

The majority of each of the laboratory's own estimated potencies lie within the 95 % limits calculated by NIBSC. Exceptions are the estimated potencies of sample D by the candidate standard antigen content assays of laboratories 03A and 03B and the in-house antigen content assays of laboratory 12, samples E and G by the candidate standard antigen content assays of laboratory 03B, sample B by the candidate standard antigen content assays of laboratories 01 and 03B and the in-house antigen content assays of laboratory 10, and sample H by the candidate standard antigen content assays of laboratory 13 and the in-house antigen content assays of laboratories 10 and 12. The cause of the discrepancies is not clear.

The overall mean potencies of each sample relative to sample A, the 1st IS, for each assay method, are shown in Table 4. There are no significant differences between the mean estimates of potency by the different antigen content assays, for any of the samples, except for sample D when the candidate standard antigen content assays give a significantly lower mean estimate than the in-house antigen content assays. For each sample, the mean estimates from the candidate standard and the in-house antigen content assays were calculated for each laboratory, to allow the comparison of potency estimates from the immunogenicity assays and the antigen content assays. There were no significant differences between the mean estimates

of potency by the immunogenicity assays and the antigen content assays, for samples C, D and B. However, the immunogenicity assays produce a significantly higher mean estimate of potency for samples E, F and H, and a significantly lower mean estimate for sample G, than the antigen content assays.

Table 2a — *Potency estimates and 95 % confidence limits for NIBSC calculations of samples C and D relative to sample A (or sample C). The potency of sample A was assumed to be 1 for both assays.*

Lab No	Assay method	Potency of C			Potency of D		
		N	95% limits	L	N	95% limits	L
01		1.30	*OA*	*NP*	7.63	0.20 - 284.40	*NP*
02		1.08	*OA*	*NP*	5.43	*OA*	*NP*
03		1.14	*OA*	*NP*	4.71	0.05 - 464.81	*NP*
04		0.67	*OA*	*NP*	1.80	0.21 - 15.38	*NP*
06		1.72	*OA*	*NP*	2.28	0.38 - 13.86	*NP*
09A		0.44	*OA*	*NP*	6.40	0.05 - 893.97	*NP*
09B	Immuno-	*NT*	*NT*	*NT*	*NT*	*NT*	*NT*
10	genicity	1.05	*OA*	*NP*	3.12	0.02 - 465.22	*NP*
11		0.94	*OA*	*NP*	7.03	2.63 - 18.79	*NP*
13		0.75	*OA*	*NP*	8.05	*OA*	*NP*
15		1.20	*OA*	*NP*	2.89	*OA*	*NP*
17A		1.89	*OA*	*NP*	2.14	*OA*	*NP*
17B		1.05	*OA*	*NP*	2.04	0.78 - 5.29	*NP*
01		*NS*	*NS*	0.93#	2.50	2.37 - 2.65	2.45
02		*NS*	*NS*	*NP*	2.66	1.76 - 4.03	*NP*
03A		*NS*	*NS*	0.89#	1.45	1.36 - 1.55	1.35
03B		*NS*	*NS*	0.87#	1.65	1.54 - 1.76	1.52
04		1.01	0.73 - 1.39	1.01	2.07	0.88 - 4.88	2.08
05		*NS*	*NS*	*NP*	2.98	2.87 - 3.09	*NP*
06	AC-CS	*NS*	*NS*	1.03#	2.52	2.30 - 2.76	2.60
07		*NS*	*NS*	*NP*	2.77	2.05 - 3.73	*NP*
08A		1.00	0.90 - 1.12	1.00	1.65	1.27 - 2.13	1.61
08B		0.97	*OA*	0.99	1.65	*OA*	1.78
09		*NS*	*NS*	*NP*	2.47	2.21 - 2.76	*NP*
10		*NS*	*NS*	1.06#	2.63	2.33 - 2.96	2.70
12		*NS*	*NS*	1.00#	2.87	2.65 - 3.10	2.84
13		*NS*	*NS*	1.04#	2.27	2.15 - 2.41	2.40
17		0.90	0.37 - 2.20	*NP*	2.31	2.19 - 2.44	*NP*
01		0.96	0.87 - 1.05	0.98	2.30	1.34 - 3.96	2.41
02A		*NS*	*NS*	*NP*	5.58	4.58 - 6.80	*NP*
02B		*NS*	*NS*	*NP*	4.83*	*OA*	4.83
04		1.03	0.90 - 1.18	0.99	5.50	3.83 - 7.90	5.40
05		*NS*	*NS*	*NP*	2.63	2.54 - 2.73	*NP*
08	AC-IH	0.98	0.89 - 1.08	1.02	1.54	1.35 - 1.77	1.58
10		*NS*	*NS*	1.05#	5.96	5.52 - 6.44	6.15
11		*NS*	*NS*	*NP*	2.35	1.73 - 3.20	*NP*
12		1.00	0.78 - 1.28	1.00	8.16	7.82 - 8.52	7.54
13		1.01	0.88 - 1.16	0.99	4.80	1.92 - 11.97	4.91
17		1.12	0.12 - 10.08	1.12	4.93	3.67 - 6.63	*NP*

AC-CS: Antigen content-candidate standard method; AC-IH: Antigen content in-house method; N: geometric mean of NIBSC individual assay potency estimates; L: geometric mean of laboratories' own individual assay potency estimates; OA: estimate of potency based on one assay only; NP: not provided; NS: standard and unknown sample not in same assay or plate; NT: sample not tested.

uncertain how the estimate was obtained since samples A and C were not assayed on the same plate.

* laboratory's own potency estimate.

Table 2b — *Potency estimates of samples E, F and G relative to sample A (or sample C), prior to desorption for the antigen content assay. Shown are 95 % confidence limits for NIBSC calculations. The potency of sample A was assumed to be 1.*

Lab No	Assay method	Potency of E			Potency of F			Potency of G		
		N	95% limits	L	N	95% limits	L	N	95% limits	L
01	Immuno-genicity	0.52	0.27 - 1.03	*NP*	4.70	*OA*	*NP*	0.25	*OA*	*NP*
02		*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
03		0.36	0.02 - 7.29	*NP*	1.95	*OA*	*NP*	0.76	*OA*	*NP*
04		0.33	0.13 - 0.86	*NP*	1.88	*OA*	*NP*	0.11	*OA*	*NP*
06		0.61	0.58 - 0.64	*NP*	3.05	*OA*	*NP*	0.15	*OA*	*NP*
09A		0.53	0.01 – 53.29	*NP*	1.22	*OA*	*NP*	0.35	*OA*	*NP*
09B		*NT*	*NT*	*NP*	1.99	*OA*	*NP*	0.19	*OA*	*NP*
10		0.38	0.00 – 63.72	*NP*	0.89	*OA*	*NP*	0.14	*OA*	*NP*
11		0.33	0.01 - 8.57	*NP*	1.78	*OA*	*NP*	0.47	*OA*	*NP*
13		0.45	0.04 - 5.19	*NP*	1.69	*OA*	*NP*	0.23	*OA*	*NP*
15		0.18	0.07 - 0.43	*NP*	0.67	*OA*	*NP*	0.32	*OA*	*NP*
17A		0.31	0.00 – 239.32	*NP*	4.02	*OA*	*NP*	0.30	*OA*	*NP*
17B		0.27	0.00 – 104.69	*NP*	1.98	*OA*	*NP*	0.10	*OA*	*NP*
01	AC-CS	0.31	0.24 - 0.41	0.30	1.28	1.09 - 1.51	1.21	0.39	0.35 - 0.43	0.40
02		0.12	0.06 - 0.26	*NP*	1.55	0.43 - 5.59	*NP*	0.55	0.49 - 0.62	*NP*
03A		0.29	0.26 - 0.32	0.27	1.37	1.17 - 1.60	1.29	0.42	0.38 - 0.45	0.39
03B		0.29	0.28 - 0.30	0.27	1.27	1.04 - 1.54	1.20	0.42	0.39 - 0.44	0.38
04		*NS*	*NS*	0.33#	*NS*	*NS*	*NP*	*NS*	*NS*	0.41#
05		0.31	0.28 - 0.34	*NP*	1.31	1.19 - 1.44	*NP*	0.45	0.42 - 0.49	*NP*
06		0.29	0.24 - 0.34	0.30	1.32	1.20 - 1.45	1.26	0.40	0.37 - 0.43	0.40
07		0.29	0.20 - 0.40	*NP*	1.21	0.99 - 1.49	*NP*	0.43	0.36 - 0.51	*NP*
09		0.27	0.23 - 0.32	*NP*	0.96	0.80 - 1.16	*NP*	0.42	0.34 - 0.51	*NP*
10		0.25	0.22 - 0.28	0.26	0.97	0.83 - 1.13	1.05	0.32	0.20 - 0.51	0.33
13		0.20	0.20 - 0.21	0.20	0.64	0.58 - 0.72	0.71	0.38	0.35 - 0.42	0.39
01	AC-IH	0.30	0.28 - 0.32	0.30	1.06	0.96 - 1.16	1.06	0.39	0.37 - 0.41	0.39
02A		0.38	0.21 - 0.67	*NP*	2.12	1.39 - 3.22	*NP*	0.91	0.74 - 1.11	*NP*
02B		0.12*	*OA*	0.12	1.26*	*OA*	1.26	0.73*	*OA*	0.73
04		*NS*	*NS*	0.32#	*NS*	*NS*	1.25#	*NS*	*NS*	0.38#
05		0.28	0.26 - 0.29	*NP*	1.20	1.00 - 1.45	*NP*	0.47	0.45 - 0.49	*NP*
10		0.27	0.20 - 0.37	0.29	1.22	1.13 - 1.32	1.24	0.34	0.27 - 0.43	0.38
11		0.17	0.12 - 0.24	*NP*	0.44	0.40 - 0.48	*NP*	0.36	0.24 - 0.52	*NP*
13		0.17	0.13 - 0.21	0.17	0.75	0.47 - 1.19	0.84	0.50	*OA*	0.46

AC-CS: Antigen content-candidate standard method; AC-IH: Antigen content in-house method; N: geometric mean of NIBSC individual assay potency estimates; L: geometric mean of laboratories' own individual assay potency estimates; OA: estimate of potency based on one assay only; NP: not provided; NS: standard and unknown sample not in same assay or plate; NT: sample not tested.

uncertain how the estimate was obtained since unknown and standard samples were not assayed on the same plate.

* laboratory's own potency estimate.

Table 2c — *Potency estimates and 95% confidence limits for NIBSC calculations of samples B and H relative to sample A (or sample C). The potency of sample A was assumed to be 1 for both assays.*

Lab No	Assay method	Potency of B			Potency of H		
		N	95% limits	L	N	95% limits	L
01	Immuno-genicity	*TL*	*TL*	*NP*	2.36	*OA*	*NP*
02		*TL*	*TL*	*NP*	*NS*	*NS*	*NS*
03		*TL*	*TL*	*NP*	3.26	*OA*	*NP*
04		0.047	*OA*	*NP*	0.42	*OA*	*NP*
06		0.037	*OA*	*NP*	0.59	*OA*	*NP*
09A		0.092	*OA*	*NP*	1.72	*OA*	*NP*
09B		*NT*	*NT*	*NT*	0.41	0.07 - 2.40	*NP*
10		0.045	*OA*	*NP*	1.24	*OA*	*NP*
11		0.069	*OA*	*NP*	1.03	*OA*	*NP*
13		0.13	*OA*	*NP*	1.74	*OA*	*NP*
15		0.021	*OA*	*NP*	0.41	*OA*	*NP*
17A		*NT*	*NT*	*NT*	0.73	*OA*	*NP*
17B		0.011	*OA*	*NP*	0.50	*OA*	*NP*
01	AC-CS	0.053	0.051 - 0.056	0.047	0.098	0.074 - 0.13	0.10
02		0.084	0.027 - 0.26	*NP*	0.080	0.054 - 0.12	*NP*
03A		0.053	0.048 - 0.058	0.049	0.21	0.14 - 0.32	0.19
03B		0.052	0.049 - 0.055	0.048	0.075	0.043 - 0.13	0.063
04		0.055	0.025 - 0.12	0.070	*NS*	*NS*	0.26#
05		0.063	0.055 - 0.072	*NP*	0.39	0.37 - 0.41	*NP*
06		0.057	0.055 - 0.060	0.10	0.094	0.084 - 0.11	0.10
07		0.055	0.034 - 0.090	*NP*	0.082	0.059 - 0.11	*NP*
08A		0.067	0.046 - 0.099	0.083	0.11	0.094 - 0.12	0.11
08B		*NT*	*NT*	*NT*	0.47	*OA*	0.42
09		0.064	0.056 - 0.073	*NP*	0.085	0.059 - 0.13	*NP*
10		0.058	0.049 - 0.070	0.055	0.078	0.058 - 0.10	0.074
12		0.059	0.058 - 0.060	0.058	0.10	0.092 - 0.12	0.10
13		0.055	0.051 - 0.059	0.057	0.073	0.067 - 0.080	0.085
17		0.050	0.047 - 0.053	*NP*	0.088	0.081 - 0.096	*NP*
01	AC-IH	0.057	0.050 - 0.065	0.056	0.077	0.068 - 0.088	0.077
02A		*NT*	*NT*	*NT*	0.25	0.15 - 0.40	*NP*
02B		0.05*	*OA*	0.058	0.24*	*OA*	0.24
04		0.051	0.030 - 0.086	0.050	*NS*	*NS*	0.20#
05		0.041	0.038 - 0.045	*NP*	0.20	0.17 - 0.24	*NP*
08		0.062	0.055 - 0.069	0.057	0.47	0.32 - 0.70	0.49
10		0.10	0.089 - 0.12	0.059	0.23	0.21 - 0.24	0.26
11		0.047	0.032 - 0.069	*NP*	0.047	0.021 - 0.10	*NP*
12		0.057	0.054 - 0.060	0.056	0.13	0.10 - 0.17	0.18
13		0.058	0.052 - 0.065	0.059	*NS*	*NS*	0.42
17		0.072	0.060 - 0.088	*NP*	0.32	0.24 - 0.44	*NP*

AC-CS: Antigen content-candidate standard method; AC-IH: Antigen content in-house method; N: geometric mean of NIBSC individual assay potency estimates; L: geometric mean of laboratories' own individual assay potency estimates; OA: estimate of potency based on one assay only; NP: not provided; NS: standard and unknown sample not in same assay or plate; NT: sample not tested; TL: responses too low to obtain an estimate.

uncertain how the estimate was obtained since samples A and C were not assayed on the same plate.

* laboratory's own potency estimate.

• Figures 1a-1g •

Legend: Assay Method — Antigenicity (CS) — Antigenicity (IH) — Immunogenicity

Figure 1a — *Estimates of potency of sample C relative to sample A, the 1st IS. Estimates are expressed on a log_{10} scale. The potency of sample A was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

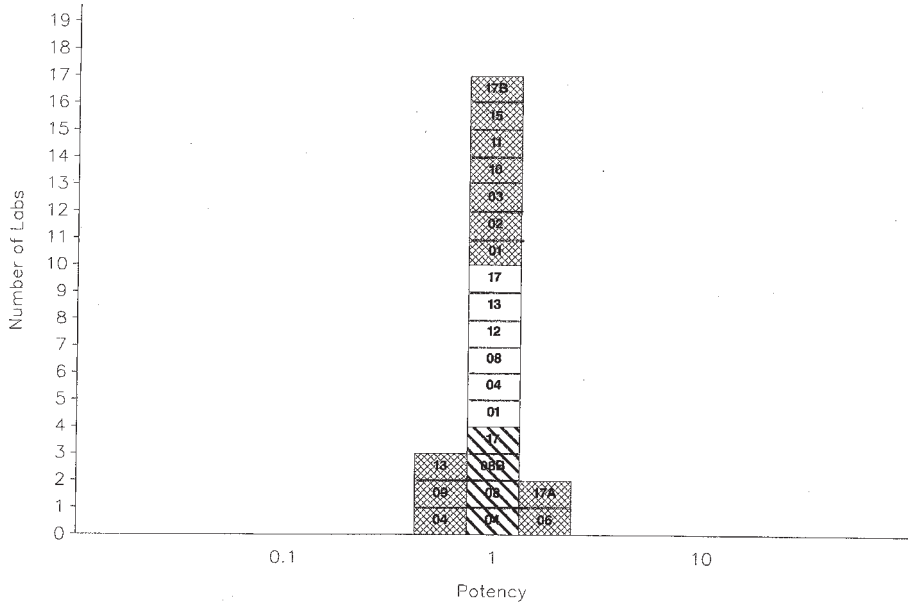


Figure 1b — *Estimates of potency of sample D relative to sample A, the 1st IS. Estimates are expressed on a log_{10} scale. The potency of sample A was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

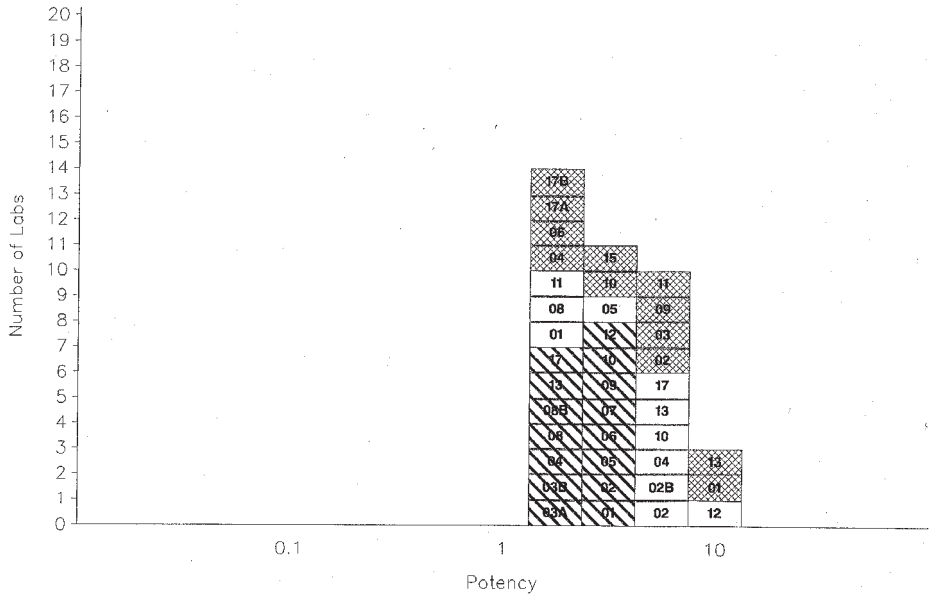


Figure 1c — *Estimates of potency of sample E relative to sample A, the 1st IS. Estimates are expressed on a $\log_{10}$ scale. The potency of sample A was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

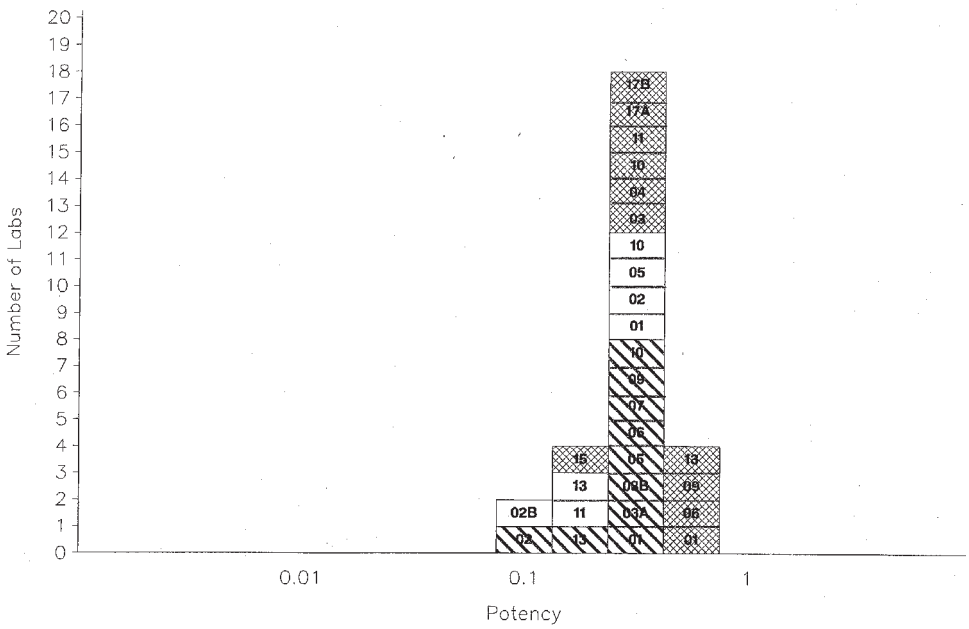


Figure 1d — *Estimates of potency of sample F relative to sample A, the 1st IS. Estimates are expressed on a $\log_{10}$ scale. The potency of sample A was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

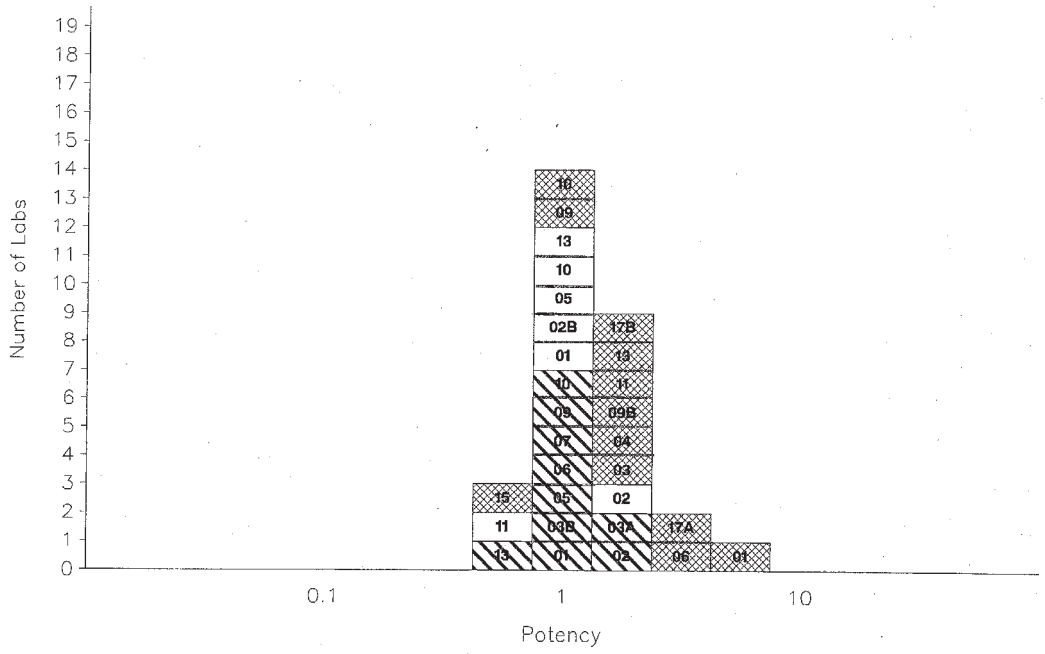


Figure 1e — *Estimates of potency of sample G relative to sample A, the 1st IS. Estimates are expressed on a log_{10} scale. The potency of sample A was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

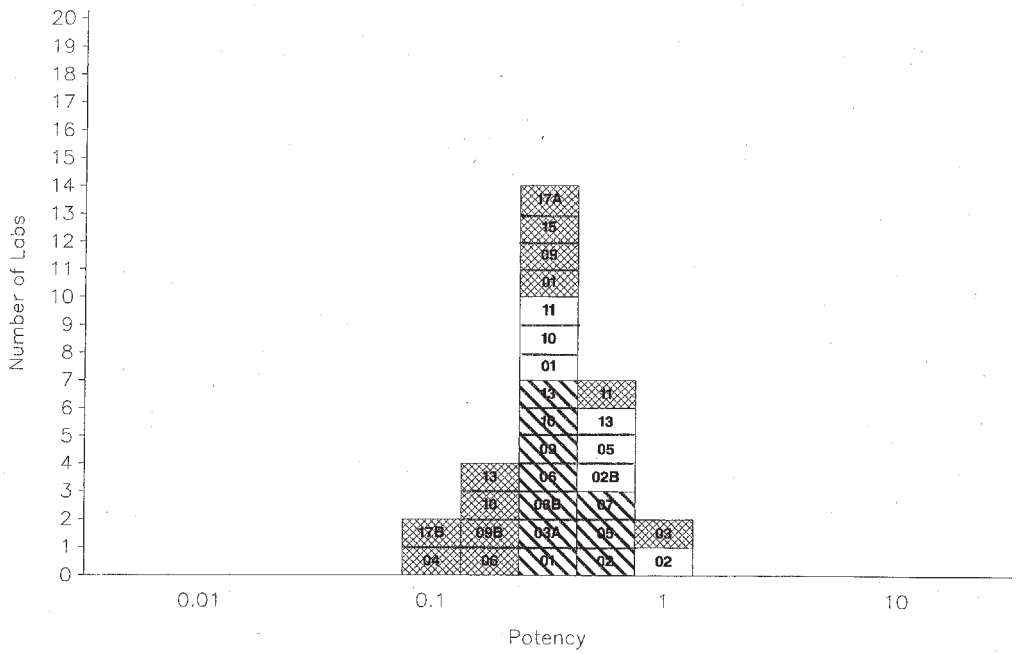


Figure 1f — *Estimates of potency of sample B relative to sample A, the 1st IS. Estimates are expressed on a log_{10} scale. The potency of sample A was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

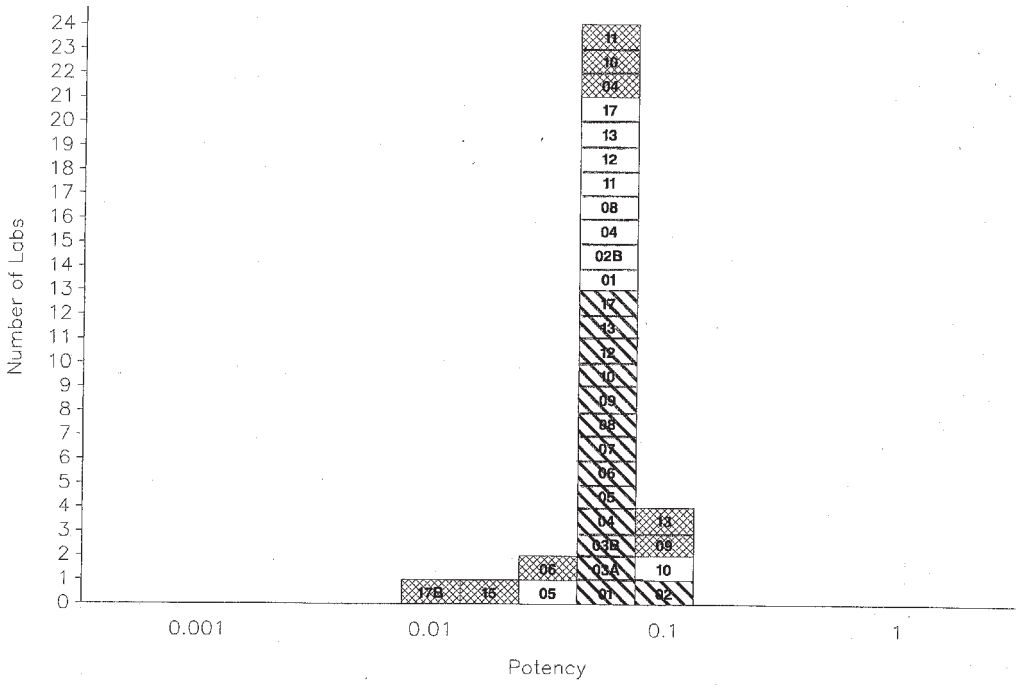
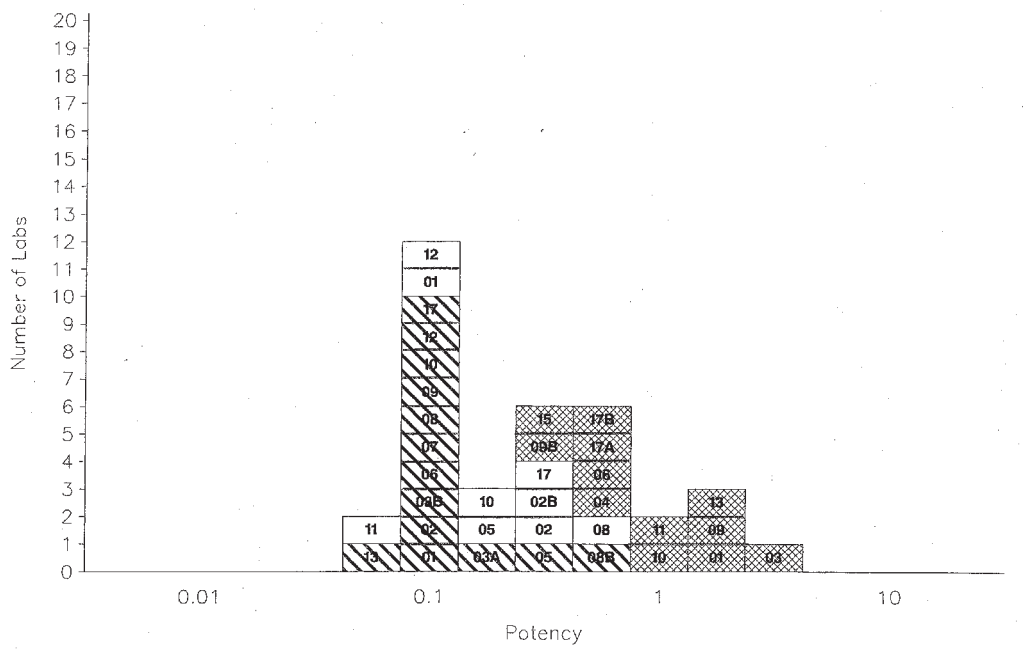


Figure 1g — *Estimates of potency of sample H relative to sample A, the 1st IS. Estimates are expressed on a log_{10} scale. The potency of sample A was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*



Because samples A and C are identical, the potency estimate of sample C relative to sample A should equal 1. All of the laboratories which performed the antigen content assays, both the candidate standard method or their own in-house method, obtained a potency estimate within 12 % of this value, even if the laboratories' own estimates are used, as shown in Table 2a. All of the laboratories that performed the immunogenicity assays obtained a potency estimate within 35 % of this value, with the exception of laboratories 09A and 17A whose estimates were within 56 % and 89 %, respectively. However, in the case of the immunogenicity assays, the estimates of potency of sample C are based on one assay only. Nevertheless, the geometric means of all potency estimates were 0.97, 1.01 and 1.03 for the candidate standard and in-house antigen content assays and the immunogenicity assays, respectively (Table 4).

Sample A was manufactured in an identical manner to sample F except for the omission of the usual alum adjuvant. If alum adjuvant has no effect on either immunogenicity or antigen content then the potency of F relative to A should equal 1. The geometric mean potency estimates were 1.87, 1.16 and 1.05 for the immunogenicity, the candidate standard antigen content and in-house antigen content assays respectively (Table 4). This difference is not significant for the antigen content assays but is for the immunogenicity assays ($p<0.005$).

5.5. INTRA-LABORATORY VARIABILITY

The variability within each laboratory, expressed as % GCV's for each sample, separately for each assay method, is given in Table 3. It should be noted that, for the immunogenicity assays, this can only be calculated for samples D and E since the potency estimates of the other samples are based on one assay only, from each laboratory. In general, for samples D and E, the immunogenicity assays produce higher intra-laboratory variability than the antigen content assays. For the Ph. Eur. BRP candidates, the variability within each laboratory is highest for samples E and H and lowest for sample G.

Table 3 — *Intra-laboratory variability (between-assay GCV's %). Sample A (or sample C) as standard (based on NIBSC calculations).*

Lab No	Assay method	C	D	E	F	G	B	H
01	Immuno-genicity	*OA*	49.6	7.8	*OA*	*OA*	*TL*	*OA*
02		*OA*	*OA*	*NS*	*NS*	*NS*	*TL*	*NS*
03		*OA*	66.7	39.6	*OA*	*OA*	*TL*	*OA*
04		*OA*	27.0	11.3	*OA*	*OA*	*OA*	*OA*
06		*OA*	22.2	0.6	*OA*	*OA*	*OA*	*OA*
09A		*OA*	73.3	67.2	*OA*	*OA*	*OA*	*OA*
09B		*OA*	*NT*	*NT*	*OA*	*OA*	*NT*	21.6
10		*OA*	74.6	76.8	*OA*	*OA*	*OA*	*OA*
11		*OA*	11.6	43.8	*OA*	*OA*	*OA*	*OA*
13		*OA*	*OA*	31.4	*OA*	*OA*	*OA*	*OA*
15		*OA*	*OA*	10.3	*OA*	*OA*	*OA*	*OA*
17A		*OA*	*OA*	109.4	*OA*	*OA*	*NT*	*OA*
17B		*OA*	11.2	94.3	*OA*	*OA*	*OA*	*OA*
01	AC-CS	*NS*	5.4	23.5	13.9	8.3	3.9	18.5
02		*NS*	48.4	62.4	67.6	7.1	57.3	28.8
03A		*NS*	6.5	8.6	13.3	6.9	8.3	30.9
03B		*NS*	6.5	3.8	17.1	5.2	4.9	41.2
04		13.9	41.2	*NS*	*NS*	*NS*	36.4	*NS*
05		*NS*	3.6	8.4	7.8	6.6	11.5	3.0
06		*NS*	9.0	15.4	8.1	5.9	3.1	7.1
07		*NS*	33.1	32.4	18.1	16.0	48.5	14.0
08A		4.5	11.0	*NT*	*NT*	*NT*	16.7	5.2
08B		*OA*	*OA*	*NT*	*NT*	*NT*	*NT*	*OA*
09		*NS*	11.2	15.6	16.3	18.7	11.7	26.8
10		*NS*	12.1	10.2	13.2	45.9	15.6	20.0
12		*NS*	7.7	*NT*	*NT*	*NT*	1.8	7.3
13		*NS*	5.6	2.4	9.2	8.3	6.5	5.6
17		10.4	5.4	*NT*	*NT*	*NT*	4.8	8.4
01	AC-IH	1.0	6.2	6.7	9.6	5.6	1.5	12.5
02A		*NS*	20.7	43.5	18.4	13.8	*NT*	21.9
02B		*NS*	*OA*	*OA*	*OA*	*OA*	*OA*	*OA*
04		5.5	15.7	*NS*	*NS*	*NS*	23.6	*NS*
05		*NS*	3.6	3.5	16.4	4.1	7.0	9.9
08		3.8	5.6	*NT*	*NT*	*NT*	4.8	17.1
10		*NS*	7.7	27.7	6.5	19.7	11.9	5.0
11		*NS*	33.9	31.5	7.5	36.1	37.1	65.1
12		10.3	5.2	*NT*	*NT*	*NT*	5.8	32.0
13		5.8	10.7	9.5	20.5	*OA*	4.6	*NS*
17		27.8	32.6	*NT*	*NT*	*NT*	16.9	34.5

AC-CS: Antigen content-candidate standard method; AC-IH: Antigen content in-house method; OA: estimate of potency based on one assay only; NS: standard and unknown sample not in same assay or plate; NT: sample not tested; TL: responses too low to obtain an estimate.

5.6. INTER-LABORATORY VARIABILITY

Inter-laboratory variability of the potency estimates of samples C, D, E, F, G, B and H relative to sample A, the 1st IS, is summarised as % GCV's in Table 4. Variability between laboratories ranged from 5.3 to 119.4. The variability between laboratories is highest for the immunogenicity assays, for all samples, except sample E. This is not surprising since the estimates of potency of all samples, except samples D and E, are based on one assay only, for each laboratory and animal immunogenicity assays are expected to be more variable than *in vitro* assays. The inter-laboratory variability is lowest for the candidate standard antigen content assays for all samples, except sample C where the inter-laboratory variability is similar for the candidate standard and in-house antigen content assays. For the Ph. Eur. BRP candidates, the variability between laboratories is generally highest for sample H and lowest for sample E.

Table 4 — *Summary statistics of samples B, C, D, E, F, G and H relative to sample A (based on NIBSC calculations). The potency of sample A was assumed to be 1.*

Sample	Assay method	Number of laboratories	Mean potency	Mean intra-laboratory variability	Inter-laboratory variability
C	Immunogenicity	12	1.03	*OA*	49.1
	Antigen content (CS)	4	0.97	9.6	5.3
	Antigen content (IH)	6	1.01	9.0	5.4
D	Immunogenicity	12	3.87	42.0	76.1
	Antigen content (CS)	15	2.24	14.8	26.2
	Antigen content (IH)	11	3.96	14.2	67.5
E	Immunogenicity	11	0.37	44.8	42.0
	Antigen content (CS)	10	0.25	18.3	33.7
	Antigen content (IH)	7	0.23	20.4	48.8
F	Immunogenicity	12	1.87	*OA*	75.7
	Antigen content (CS)	10	1.16	18.5	28.8
	Antigen content (IH)	7	1.05	13.1	63.0
G	Immunogenicity	12	0.24	*OA*	83.9
	Antigen content (CS)	10	0.41	12.9	15.0
	Antigen content (IH)	7	0.49	15.9	45.0
B	Immunogenicity	8	0.044	*OA*	119.4
	Antigen content (CS)	14	0.058	16.5	14.4
	Antigen content (IH)	10	0.059	12.6	28.1
H	Immunogenicity	12	0.94	21.6	107.9
	Antigen content (CS)	14	0.12	16.7	84.2
	Antigen content (IH)	9	0.18	24.7	105.3

CS: candidate standard method; IH: in-house method; OA: estimates of potency based on one assay only.

It should be borne in mind that vaccines manufactured in identical ways to these samples have all been shown to be efficacious in humans and are licensed for use. The potency of each vaccine was established as in-house units during licensing, on the basis of clinical trials. The given activities are dependent on the assay technique used for determination and hence do not reflect the clinical potency or strength of the vaccines.

5.7. COMPARISON OF THE PH. EUR. BRP CANDIDATES

In general, all of the Ph. Eur. BRP samples are linear and parallel with one another and with the 1st IS, except sample H which is non-parallel to all other samples. When using the 1st IS as the standard, there are significant differences between the potency estimates obtained from the immunogenicity and antigen content assays, for all samples. However, this difference is more marked for sample G. For the antigen content assays, variability within laboratories is highest for sample E and lowest for sample G.

The summary statistics of samples F, G and H relative to sample E, and of samples E, G and H relative to sample F and of samples E, F and H relative to sample G are shown in Tables 5, 6 and 7, respectively (assuming each sample has a potency of 1 when used as the standard). Laboratory mean potency estimates of the Ph. Eur. BRP samples relative to samples E, F and G are shown in histogram form in Figures 2, 3 and 4, respectively.

For the antigen content assays, variability between laboratories based on the estimates relative to sample F is, in general, lower for all samples than that based on estimates relative to sample E or sample G. For the immunogenicity assays, the potencies of samples G and H could not be estimated relative to sample E, since they were not included in the same assay. Therefore, meaningful comparisons of the variability between laboratories, using the different Ph. Eur. BRP samples as standards, cannot be made for either the immunogenicity assays or overall.

Samples G and H were not tested in the same immunogenicity assay as sample E. However, for sample F relative to sample E, there are no significant differences between the potency

estimates from the immunogenicity and antigen content assays. Using sample F or sample G as the standard, there are significant differences between the potency estimates from the immunogenicity and the antigen content assays.

The comparisons above suggest that there is little to choose from between samples E, F and G, as suitable Ph. Eur. BRPs. All of the samples need desorption prior to antigen content assay. A common desorption protocol was not identified. As manufacturers do not wish their methods disclosed to competitors then product specific reference materials had to be established.

The fourth candidate Ph. Eur. BRP was a formalin inactivated antigen adjuvanted in combination with influenza proteins that did not require desorption of antigen prior to antigen content assay. This sample, H, gave non-parallel dose-responses to the other Ph. Eur. BRP samples in antigen content assays. After the study was completed the manufacturer disclosed to the study organisers that the sample should have been detergent treated prior to antigen content assay. As this was not done conclusions about the antigenic content of this preparation should not be made on the basis of this study. Sample H gave parallel dose responses in the immunogenicity assays and can be assigned a unitage of 94 IU per ml of immunogenicity activity. However, the manufacturer of this sample has concerns about the long-term stability of this material and it would seem prudent not to establish this material as a BRP without further study.

Table 5 — S*ummary statistics of samples F, G and H relative to sample E. The potency of sample E was assumed to be 1.*

Sample	Assay method	Number of laboratories	Mean potency	Mean intra-laboratory variability	Inter-laboratory variability
F	Immunogenicity	12	5.09	*OA*	39.4
	AC – CS	11	6.15	13.3	78.0
	AC – IH	8	5.66	16.2	61.8
G	Immunogenicity	*NS*	*NS*	*NS*	*NS*
	AC – CS	11	2.06	18.2	57.2
	AC – IH	8	2.56	12.3	102.7
H	Immunogenicity	*NS*	*NS*	*NS*	*NS*
	AC – CS	11	0.57	32.5	79.0
	AC – IH	7	0.80	20.9	125.0

AC-CS: Antigen content - candidate standard method; AC-IH: Antigen content in-house method; OA: estimates of potency based on one assay only; NS: standard and unknown sample not in same assay or plate; see also footnote of Table 4.

Table 6 — S*ummary statistics of samples E, G and H relative to sample F. The potency of sample F was assumed to be 1.*

Sample	Assay method	Number of laboratories	Mean potency	Mean intra-laboratory variability	Inter-laboratory variability
E	Immunogenicity	12	0.20	*OA*	39.4
	AC – CS	11	0.16	13.3	78.0
	AC – IH	8	0.18	16.2	61.8
G	Immunogenicity	12	0.10	*OA*	81.2
	AC – CS	11	0.37	12.9	24.9
	AC – IH	8	0.44	13.0	42.3
H	Immunogenicity	13	0.44	*OA*	129.0
	AC – CS	11	0.093	22.9	68.2
	AC – IH	7	0.13	27.0	46.9

AC-CS: Antigen content candidate standard method; AC-IH: Antigen content in-house method; OA: estimates of potency based on one assay only; NS: standard and unknown sample not in same assay or plate; see also footnote of Table 4.

Table 7 — *Summary statistics of samples E, F and H relative to sample G. The potency of sample G was assumed to be 1.*

Sample	Assay method	Number of laboratories	Mean potency	Mean intra-laboratory variability	Inter-laboratory variability
E	Immunogenicity	*NS*	*NS*	*NS*	*NS*
	AC – CS	11	0.49	18.2	57.2
	AC – IH	8	0.39	12.3	102.7
F	Immunogenicity	12	9.96	*OA*	81.2
	AC – CS	11	2.72	12.9	24.9
	AC – IH	8	2.25	13.0	42.3
H	Immunogenicity	13	4.53	84.9	93.0
	AC – CS	11	0.27	29.9	73.5
	AC – IH	8	0.38	22.9	97.2

AC-CS: Antigen content candidate standard method; AC-IH: Antigen content in-house method; OA: estimates of potency based on one assay only; NS: standard and unknown sample not in same assay or plate; see also footnote of Table 4.

• Figures 2a-2c, 3a-3c, 4a-4c •

Legend: Assay Method ▨ Antigenicity (CS) ▭ Antigenicity (IH) ▩ Immunogenicity

Figure 2a — *Estimates of potency of sample F relative to sample E, a Ph. Eur. BRP candidate. Estimates are expressed on a $\log_{10}$ scale. The potency of sample E was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

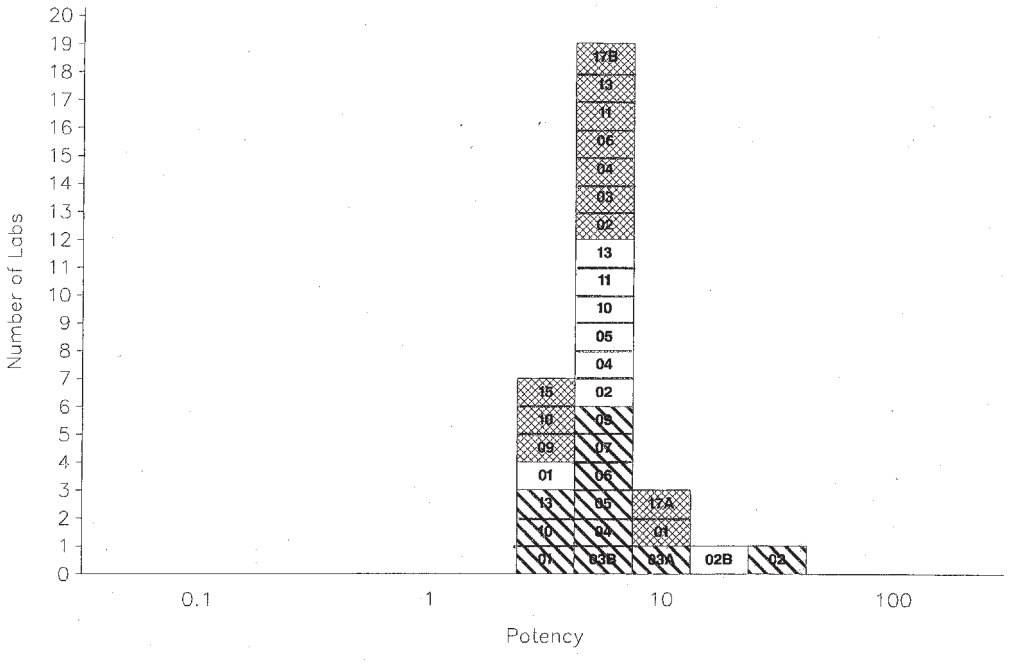


Figure 2b — *Estimates of potency of sample G relative to sample E, a Ph. Eur. BRP candidate. Estimates are expressed on a log_{10} scale. The potency of sample E was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

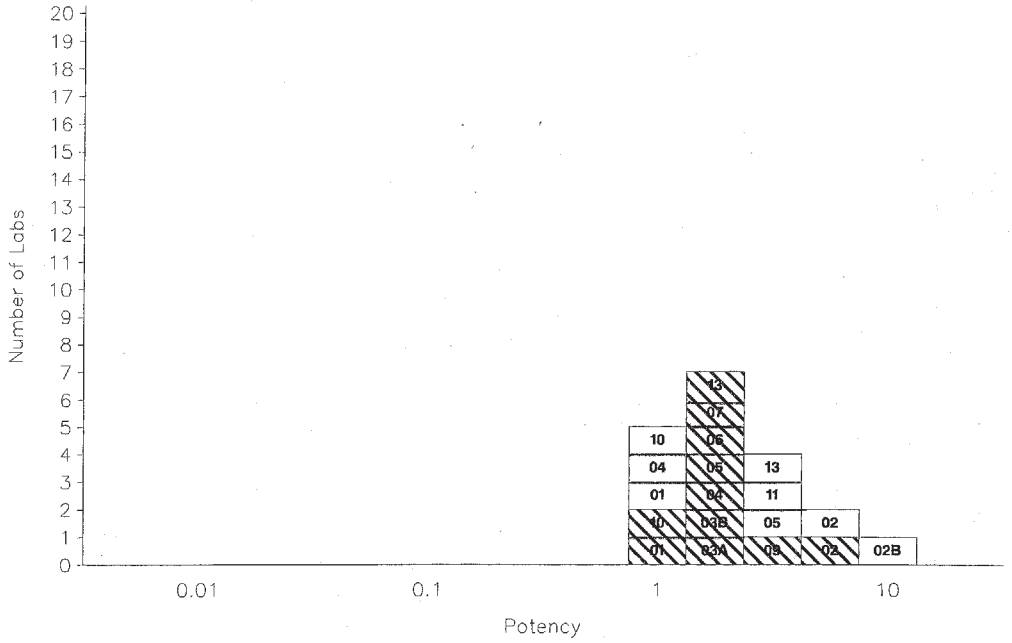


Figure 2c — *Estimates of potency of sample H relative to sample E, a Ph. Eur. BRP candidate. Estimates are expressed on a log_{10} scale. The potency of sample E was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

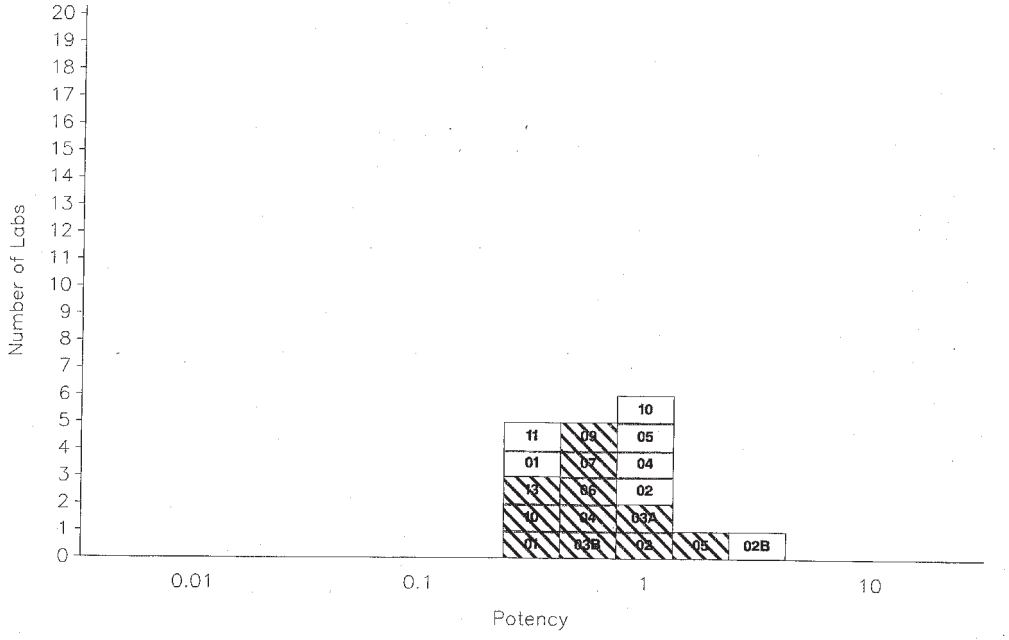


Figure 3a — *Estimates of potency of sample E relative to sample F, a Ph. Eur. BRP candidate. Estimates are expressed on a log_{10} scale. The potency of sample F was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

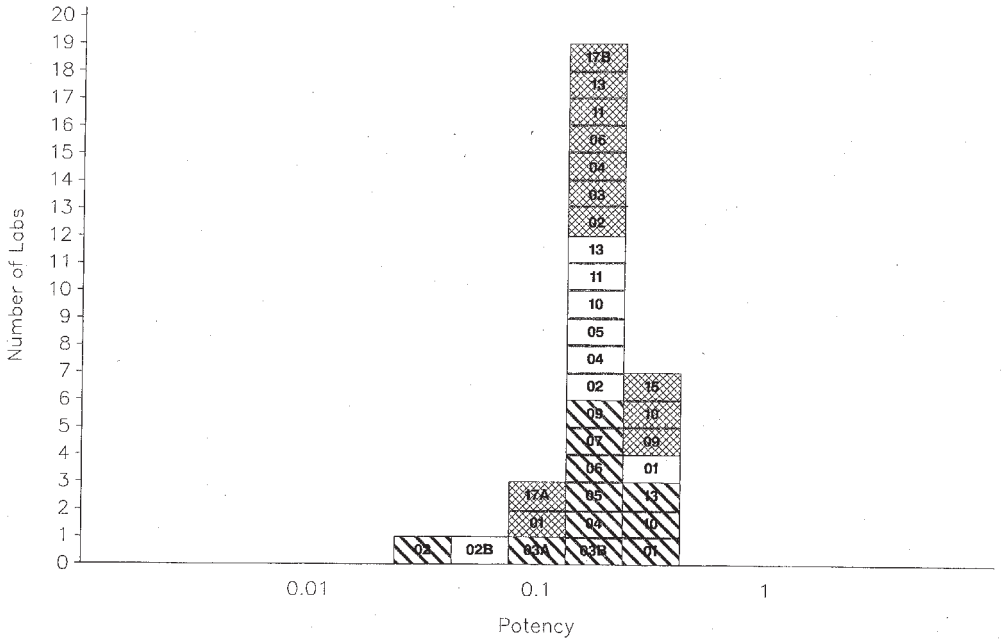


Figure 3b — *Estimates of potency of sample G relative to sample F, a Ph. Eur. BRP candidate. Estimates are expressed on a log_{10} scale. The potency of sample F was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

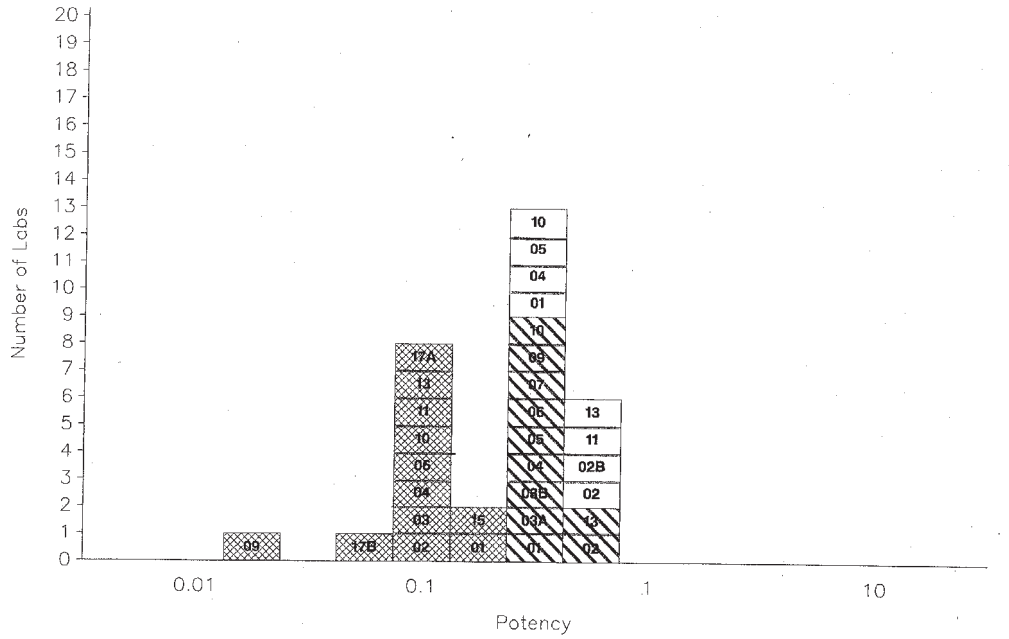


Figure 3c — *Estimates of potency of sample H relative to sample F, a Ph. Eur. BRP candidate. Estimates are expressed on a log_{10} scale. The potency of sample F was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

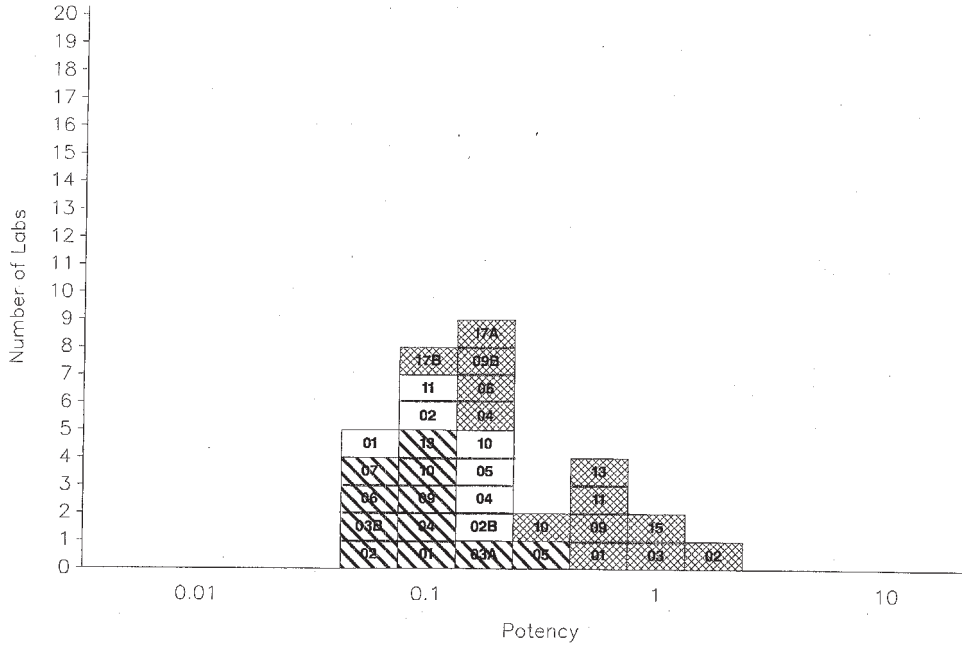


Figure 4a — *Estimates of potency of sample E relative to sample G, a Ph. Eur. BRP candidate. Estimates are expressed on a log_{10} scale. The potency of sample G was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

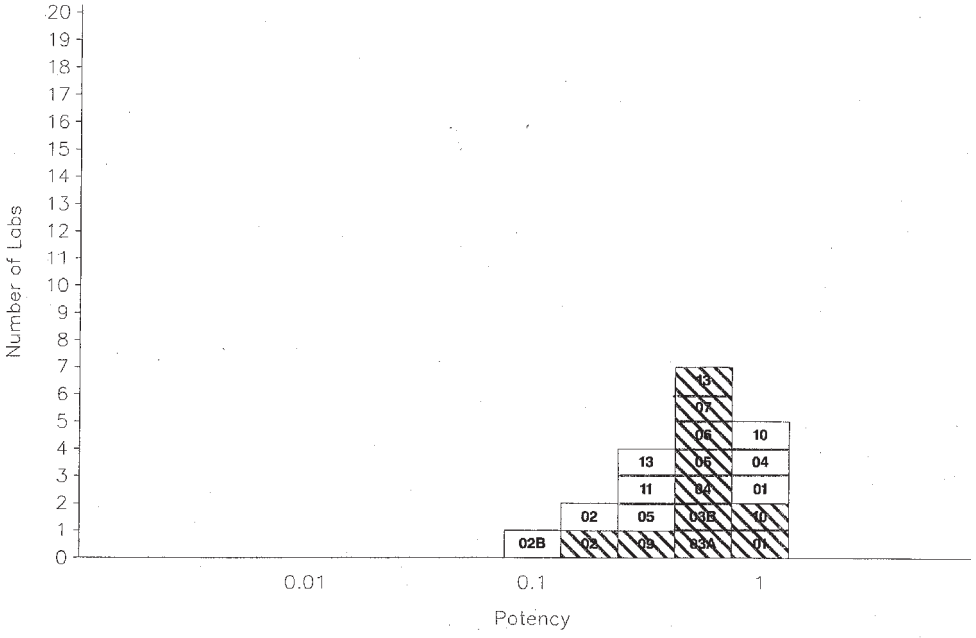


Figure 4b — *Estimates of potency of sample F relative to sample G, a Ph. Eur. BRP candidate. Estimates are expressed on a log_{10} scale. The potency of sample G was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*

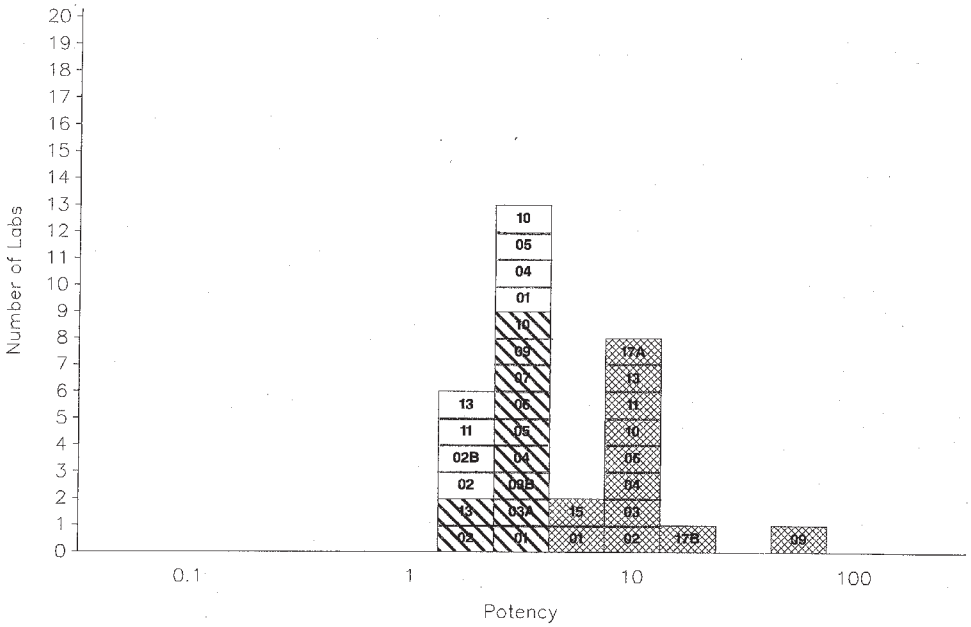
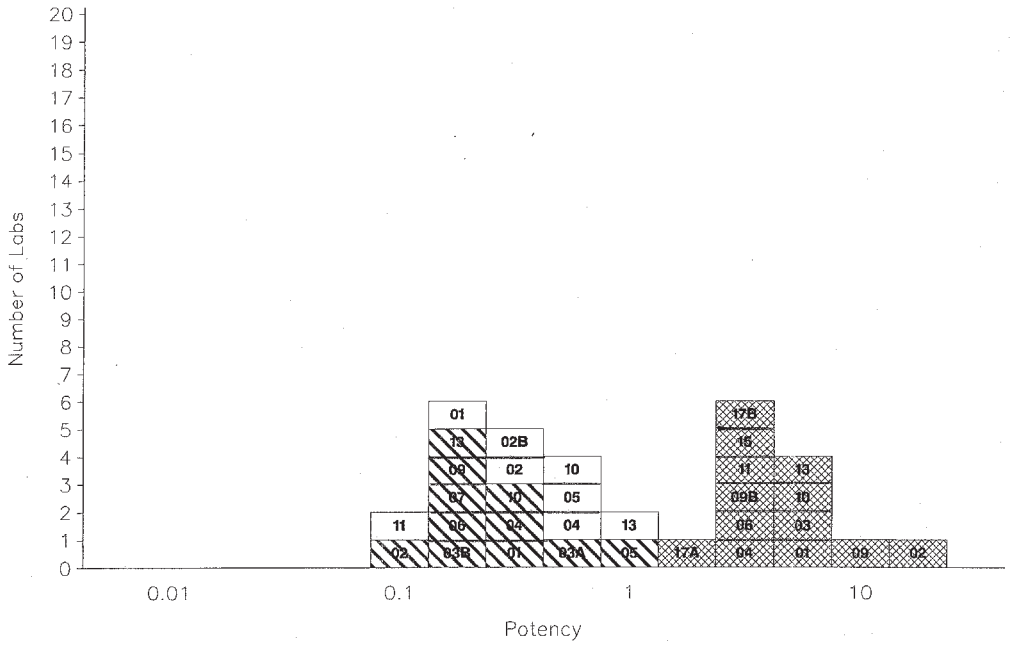


Figure 4c — *Estimates of potency of sample H relative to sample G, a Ph. Eur. BRP candidate. Estimates are expressed on a log_{10} scale. The potency of sample G was assumed to be 1. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from the laboratory. The shading represents the different assay methods.*



5.8. ASSESSMENT OF THE EDQM CANDIDATE STANDARD ANTIGEN CONTENT ASSAY

Comparing slopes of response lines across the candidate standard antigen content assays only, samples B and H were found to be significantly non-parallel to all other samples, but not to each other. However, if slopes of response lines are compared across the in-house antigen content assays only, then sample H was found to be significantly non-parallel from samples A and D, but no other significant non-parallelism is present. The percentage of valid candidate standard antigen content assays is somewhat lower than the percentage of valid in-house antigen content assays (Table 1). However, for reasons given above, it is not surprising that problems with parallelism exist for samples B and H.

Using the 1st IS as the standard, there are no significant differences between the mean estimates of potency by the different antigen content assays, for any of the samples, except for sample D when the candidate standard antigen content assays give a significantly lower mean estimate than the in-house antigen content assays (Table 4). The variability between laboratories is lower for the candidate standard antigen content assays than for the in-house antigen content assays, for all samples, except sample C where the variability between laboratories is similar for both methods. Based on these results, the candidate standard method appears to be suitable as a standard method for antigen content assays.

5.9. COMPARISON OF THE IMMUNOGENICITY AND ANTIGEN CONTENT ASSAYS

For each sample, the mean estimates from the candidate standard and the in-house antigen content assays were calculated for each laboratory, to allow the comparison of potency estimates from the immunogenicity assays and the antigen content assays. Using the 1st IS as the standard, there were no significant differences between the mean estimates of potency by the immunogenicity assays and the antigen content assays, for samples C, D and B. However, the immunogenicity assays produced a significantly higher mean estimate of potency for samples E, F and H, and a significantly lower mean estimate for sample G, than the antigen content assays. This is shown pictorially in Figure 5. For samples E, F, G and H, a similar picture is drawn irrespective of the standard used.

Figure 5 — *Estimates of potency from the antigen content assays against estimates of potency from the immunogenicity assays against sample A, the 1st IS. Estimates are expressed on a log_{10} scale. The letter represents the sample code. The line represents equality of the estimates from the antigen content assays and the immunogenicity assays.*

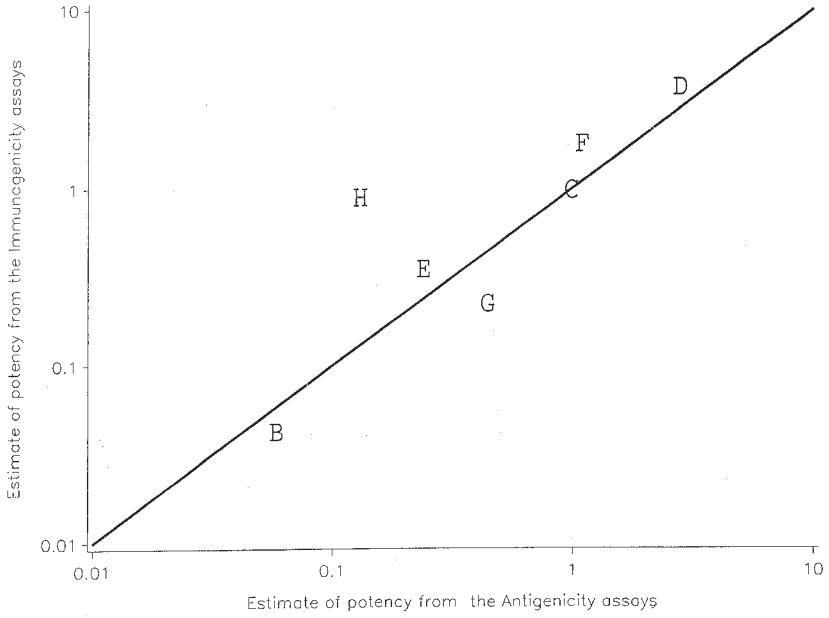


Table 8 — *Details of immunogenicity assays performed by participants*

Lab	Mouse Strain	wt range (g)	age range (weeks)	sex	vaccine diluent	IV (ml)	IR	number animals per dilution	days to bleed	sero-logical method	name of serology
01	NMRI	na	4-5	f	ng	1	i.p.	11	28	EIA	HAVAB
02	ng	ng	ng	ng	ng	ng	ng	10	ng	EIA	AXSYM
03	ICR	na	4-6	f	0.5 mg/ml alhydrogel in normal saline	1	i.p.	10	28-30	EIA	Biokit
04	NMRI	na	4-6	f	0.5 mg/ml Al in phosphate/saline buffer	1	s.c.	10	28-32	RIA	HAVAB
06	NMRI	14-18	na	f	0.05% $Al(OH)_3$	1	s.c.	15	28	RIA	HAVAB
09A	ICR	na	4-6	f	2% alhydrogel in normal saline	1	i.p.	10	28	EIA	HAVAB
09B	ICR	na	4-6	f	2% alhydrogel in PBS (sample H only)	1	i.p.	10	28	EIA	HAVAB
10	NMRI	12-14	na	f	0.6 mg/ml alum	1	s.c.	12	28-30	EIA	IMX HAVAB
11	Swiss CD1	18-20	5	f	0.5 mg/ml alhydrogel in normal saline	1	s.c.	8	29-30	EIA	Enzygnost
13	ICR	25-30	na	f	ng	1	i.p.	8	28	EIA	HAVAB
15	NIH	12-14	na	f	ng	0.5	s.c.	10	28	EIA	Enzygnost
17A	CD1	17-21	4	f	ng	0.5	s.c.	10	30-32	RIA	HAVAB
17B	CD1	17-21	4	f	ng	0.5	s.c.	10	30-32	EIA	Enzygnost

na: not available ; ng: not given ; f: female ; IV: inoculum volume ; IR: inoculation route.

6. DISCUSSION

All of the Ph. Eur. BRP samples were representative of vaccines on the market. Three were formalin inactivated alum adjuvanted antigens. All three gave linear and parallel dose responses with each other in immunogenicity assays and, after desorption of antigen with product specific protocols, antigen content assays suggesting that a single reference preparation could be used for formalin inactivated alum adjuvanted vaccines. However a common protocol for desorption of antigen was not identified. As manufacturers do not wish their desorption methods disclosed to competitors it was necessary to establish product specific reference materials. An alternative option would be to use a non-adjuvanted preparation, for example the 1st IS. However data from this study showed that the immunogenicity of non-adjuvanted antigen was significantly less than adjuvanted antigen (sample A compared to sample F). The implication of adopting a non-adjuvanted antigen would be that the pharmacopoeial potency requirement (*Hepatitis A vaccine (inactivated, adsorbed) (1998:1107)*) may need to be re-evaluated. As this is not practical, at least in the short-term, three separate and product-specific BRPs were adopted.

The fourth candidate Ph. Eur. BRP was a formalin inactivated antigen adjuvanted in combination with influenza proteins that did not require desorption of antigen prior to antigen content assay. This sample, H, gave non-parallel dose-responses to the other Ph. Eur. BRP samples in antigen content assays. After the study was completed the manufacturer disclosed to the study organisers that the sample should have been detergent treated prior to antigen content assay. As this was not done, conclusions about the antigenic content of this preparation should not be made on the basis of this study. Preparation H gave parallel dose responses in the immunogenicity assays. However concerns have been expressed about the long-term stability of this material and it would seem prudent not to establish this material as a BRP without further study. These further studies could include recalibration of sample H against the 1st IS in both antigen content and immunogenicity tests to measure the extent of any degradation of the sample during the period of storage since the end of the practical work in the collaborative study.

There were no significant differences between the mean estimates of potency by the in-house and candidate standard antigen content assays for samples E, F, G and H. All of these samples are on the market in the EU. As the variability between laboratories was lower for the candidate standard antigen content assays than for the in-house antigen content assays for all samples, except sample C where the variability between laboratories is similar for both methods, it is proposed that the candidate standard assay is accepted for use within Europe. However it should be noted that for sample D, a vaccine not licensed in Europe, the candidate standard antigen content assays give a significantly lower mean estimate than the in-house antigen content assays. This illustrates that not all inactivated hepatitis A vaccines will behave in the same way in antigen content tests. Consequently the standard antigen content test would have to be validated for use with vaccines from manufacturers not included in this study. Finally it should be noted that the candidate standard method is a commercially available kit for assay of antibodies to HAV, modified to quantify HAV antigen. It would be necessary to validate any significant changes to key reagents in the kit, especially the anti-HAV coating antibodies or the anti-HAV conjugate.

The relationship between immunogenicity and antigen content was complex. For example, using the 1st IS as the standard for samples C, D and B, there were no significant differences between the mean estimates of potency by the immunogenicity assays and the antigen content assays. However, the immunogenicity assays produced a significantly higher mean estimate of potency for samples E, F and H, and a significantly lower mean estimate for sample G, than the antigen content assays. This suggests that, for inactivated hepatitis A vaccine, antigen

content assays cannot be used to predict immunogenicity. Consequently both types of assay should be used to evaluate effects of changes to a manufacturing process. Also a batch release process that only involves antigen content tests should be periodically checked to ensure that there has been no drift in immunogenicity.

7. CONCLUSION

Four formalin-inactivated, alum-adjuvanted hepatitis A antigen preparations were evaluated in an international collaborative study to determine their suitability as Ph. Eur. BRPs. Sixteen laboratories in eleven countries assayed the candidate preparations, together with two candidate IS, a coded duplicate of one antigen and an antigen of known low potency. Participants assayed the materials with established immunogenicity assays, a candidate standard antigen content assay, proposed by EDQM, and, where available, an in-house antigen content assay. Each preparation gave linear and parallel dose responses in the immunogenicity assays but two samples were significantly non-parallel to all other samples in antigen content assays. One of these two samples was the sample of known low potency. Immunogenicity assays produced higher intra-laboratory and inter-laboratory variation than the antigen content assays. Inter-laboratory variation was lowest for the candidate standard antigen content assay. Comparison of immunogenicity and antigen content assays showed that for some samples there was a significant difference between mean potency estimates from the two types of assay. This suggests that the candidate standards should be assigned separate immunogenicity and antigen content activities.

Each candidate reference material was calibrated against the 1st IS for inactivated hepatitis A vaccine which was assigned a unitage of 100 IU per ml of immunogenicity activity and 100 IU per ml of antigen content activity by the 1999 ECBS. Sample E was established as the 1st BRP for assay of type A hepatitis A vaccines (SB-Bio)(1) with unitages of 37 IU per ml of immunogenicity activity and 24 IU per ml of antigen content. Sample F was established as the 1st BRP for assay of type B inactivated hepatitis A vaccines (Aventis Pasteur)(2) with unitages of 187 IU per ml of immunogenicity activity and 111 IU per ml of antigenic content. Sample G was established as the 1st BRP for assay of type C inactivated hepatitis A vaccines (Merck & Co.)(3) with unitages of 24 IU per ml of immunogenicity activity and 45 IU per ml of antigenic content. It should be borne in mind that vaccines manufactured in identical ways to these samples have all been shown to be efficacious in humans and are licensed for use. The potency of each vaccine was established as in-house units during licensing, on the basis of clinical trials. The given activities are dependent on the assay technique used for determination and hence do not reflect the clinical potency or strength of the vaccines.

The study also evaluated a candidate standard antigen content method. There were no significant differences between potency estimates from the candidate antigen content method and in-house antigen content methods for any of the vaccines on the market in Europe (samples E, F, G, H). However there was a difference for a vaccine, sample D, on the market in a different area. The reason for this difference is not known. Thus the candidate standard antigen content method can be recommended for vaccines represented by samples E, F, G and H. Validation studies would be required before use of the method with other products.

(1) Catalogue Nr. H0205005.

(2) Catalogue Nr. H0205010; no longer available.

(3) Catalogue Nr. H0205015.

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9. ACKNOWLEDGEMENTS

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APPENDIX 1

Standard Operating Procedure for the titration of hepatitis A vaccines using ENZYGNOST Anti-HAV kit. Version 29 January 1997.

REAGENTS REQUIRED:

PBS × 10 strength:

NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	11.5 g
KH_2PO_4	2.0 g
Distilled water	1.0 litre

pH 7.4

Store at room temperature indefinitely

Dilution Buffer:

PBS (single strength)	100.0 ml
Gelatin (0.1 %)	0.1 g

Filter sterilise and store at 4 °C for one month

Do not use if the solution appears cloudy

Enzygnost© Anti-HAV enzyme immunoassay kit

Behring Diagnostics

Product Number OQEC11

This kit is supplied for the detection of antibodies to hepatitis A virus. Reagents from this kit are used to detect hepatitis A antigen in vaccines. Store kit at 4 °C until the expiry date indicated in the kit documentation.

Kit Reagents required for this study:

Microtitration plate coated with antibodies to Hepatitis A virus antigen.

Anti-HAV/POD Conjugate and Conjugate Buffer.
Working dilution as per kit instructions.

Buffer/Substrate TMB.
Working dilution as per kit instructions.

Stopping Solution POD: 0.5N Sulphuric acid as supplied with the kit.
Washing Solution POD (×20 concentrate). Dilute as per kit instructions.

PROCEDURE:

1. If necessary desorb vaccine samples as per instructions.
   Produce a record sheet for each test following the standard plate layout.

2. In dilution buffer make two to four independent series of dilutions of each vaccine to be tested, as specified for the standard plate layout. Starting dilutions for each antigen are given separately.

3. Add 100 µl of each dilution to a single well of the plate, following the layout of the record sheet.

4. Seal the plate and incubate at 3 ± 1 °C in a humid atmosphere for 2 hours ± 5 minutes.

5. Wash the plate 4 times with single-strength washing solution.

6. Immediately before use, prepare the working strength anti-HAV/POD conjugate. Add 100 µl conjugate per well. Seal the plate and incubate at 37 ± 1 °C in a humid atmosphere for 1 hour ± 5 minutes.

7. Wash the plate 4 times.

8. Immediately before use, prepare the substrate. Add 100 µl per well. Incubate at room temperature (18 to 25 °C) in the dark for 30 ± 2 minutes.

9. Stop the reaction with 100 µl per well of stopping solution.

10. Read the plate at 450 nm.

ANALYSIS OF DATA:

1. Transcribe the OD results from the spectrophotometer printout to the record sheets.

2. Calculate the potency of each preparation relative to preparation A by parallel line analysis. Assume that preparation A has a value of 1.0. Check the computer analysis printout against the spectrophotometer printout for transcription errors.

VALIDATION OF ASSAYS:

1. The test is validated by the statistical analysis. The dose response curves should be linear and parallel at the 1 % level of significance.