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The European Sero-Epidemiology Network: standardizing the enzyme immunoassay results for measles, mumps and rubella

N. ANDREWS[1], R. G. PEBODY[1], G. BERBERS[2], C. BLONDEAU[3], P. CROVARI[4], I. DAVIDKIN[5], P. FARRINGTON[11], F. FIEVET-GROYNE[3], G. GABUTTI[4,10], E. GERIKE[6], C. GIORDANO[4], L. HESKETH[8], T. MARZEC[2], P. MORGAN-CAPNER[8], K. OSBORNE[1], A. M. PLEISNER[7], M. RAUX[3], A. TISCHER[6], U. RUDEN[9], M. VALLE[5] AND E. MILLER[1]*

[1] *PHLS, Communicable Disease Surveillance Centre, 61 Colindale Avenue, London NW9 5EQ, UK*
[2] *National Institute of Public Health and the Environment, Bilthoven, The Netherlands*
[3] *Pasteur-Merieux Connaught, Paris, France*
[4] *Dept. of Health Sciences-Hygiene and Preventive Medicine Section, Faculty of Medicine, University of Genoa, Italy*
[5] *National Public Health Institute, Helsinki, Finland*
[6] *Robert Koch Institute, Berlin, Germany*
[7] *Statens Serum Institut Copenhagen, Denmark*
[8] *Preston PHL, Preston, UK*
[9] *Swedish Institute of Infectious Disease Control, Stockholm, Sweden*
[10] *Lab of Hygiene, Dept of Biology, Faculty of Science, University of Lecce, Italy*
[11] *Department of Statistics, the Open University, Milton Keynes, UK*

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SUMMARY

The ESEN (European Sero-Epidemiology Network) project was established to harmonize the seroepidemiology of five vaccine preventable infections including measles, mumps and rubella in eight European countries. This involved achieving comparability both in the assay results from testing in different centres and also sampling methodology. Standardization of enzyme immunoassay results was achieved through the development of common panels of sera by designated reference centres. The panels were tested at the reference laboratory and then distributed to each participating laboratory for testing using their routine methods. Standardization equations were calculated by regressing the quantitative results against those of the reference laboratory. Our study found large differences in unitage between participants, despite all using an EIA method standardized against an international or local standard. Moreover, our methodology adjusted for this difference. These standardization equations will be used to convert the results of main serosurvey testing into the reference country unitage to ensure inter-country comparability.

INTRODUCTION

The European Sero-Epidemiology Network (ESEN) was established in 1996 with funding from the European Union (EU) to co-ordinate and harmonize the serological surveillance for immunity to communicable diseases in Europe [1]. The project has involved serosurveys in eight collaborating countries to measure the age-specific prevalence of antibodies to five vaccine preventable infections (measles, mumps, rubella, pertussis and diphtheria). National banks of several thousand age and sex stratified sera have been collected and tested by enzyme immunoassay (EIA)

* Author for correspondence.

for antibodies to the various antigens by a national laboratory in each country.

Comparison of seroprevalence data generated by different countries is dependent upon the interchange of comparable information [2]. Variation in both the sensitivity and specificity of EIAs is well-recognized [3]. To ensure direct comparability of quantitative and qualitative antibody results generated from a variety of assays from different countries, a novel method of standardization involving several countries was developed as part of the project [1]. The results will allow a comparison of the epidemiology of these infections under different vaccination programmes. This paper outlines the methodology and results of this standardization process for measles, mumps and rubella antibody testing.

MATERIALS AND METHODS

Overall methodology

The aim of the standardization procedure was to allow direct comparisons between assay results of different countries. The process was co-ordinated from the Public Health Laboratory Service (PHLS), Communicable Disease Surveillance Centre (CDSC), London. A reference centre was selected for each antigen, with responsibility for creating and distributing a panel of specially selected sera to the other participating countries. This panel was tested by each country with their established assay method. The same assay method was then used to test the main serum bank.

For the purposes of standardization, the quantitative results of antibody testing for each antigen from each country were calibrated against those of the reference country by CDSC. 'Standardization equations' were produced for each antigen to enable conversion of the results of the participating countries to the units of the reference country. These equations were then applied to convert the main serum bank results from each participating country to standard reference laboratory units.

Participants

There were six original participating countries in ESEN: Denmark, France, Germany, Italy, The Netherlands and the United Kingdom. Finland and Sweden joined the project at a later date. Australia also participated, but only in the panel testing section.

Standard panel construction

A reference laboratory was designated for measles (Statens Serum Institut, Copenhagen, Denmark), mumps (Robert Koch Institute, Berlin, Germany) and rubella (Preston PHLS, Preston, UK). Each reference centre was responsible for constructing a panel of approximately 150 sera that included negative, equivocal (or 'low positive') and positive specimens. The composition of the panels is shown in Table 1. An international standard was included with the measles and rubella panels and a working standard developed by the designated reference centre was incorporated in the mumps panel. The rubella standard was included in the panel as a twofold pre-dilution series in phosphate buffered saline (PBS) and negative serum to enable an inter-country comparison of standard dilution curves. From each original panel, approximately 70 μl of each specimen were aliquoted to create panels for measles and 100 μl for mumps and rubella which were then distributed to the other ESEN participants.

Panel distribution

All panels were sent by courier post from the reference centre to the participating countries. The mumps and measles panels were frozen. Panels were then stored at $-20\,^{\circ}C$ until testing. Drying out of panels had occurred for two panel recipients – Finland and France. In France, the rubella panel was discarded and a repeat panel provided; whereas in Finland the measles panel was reconstituted (see later) as no further replacement panel was available at this stage of the project.

Procedures in participating countries

Each of the participants tested the panel using the EIA method of their choice (Table 2) to produce a quantitative result of specific antibody concentration calibrated against the standard serum. All countries using the Behring EIA worked with a single dilution (1:231) to produce a quantitative result [4, 5]. This value was expressed in international units for measles and rubella and in titres for mumps. A calibration against the mumps working standard was also performed by some countries and the value expressed in arbitrary units. Those countries utilizing other commercial kits or in-house assays used dilution curves of sera calibrated against an in-house standard

Table 1. *Composition of reference serum panels for measles, mumps and rubella*

	Antigen		
	Measles	Mumps	Rubella
Reference laboratory	Denmark SSI, Copenhagen	Germany RKI, Berlin	UK PHL, Preston
Panel size	137	150	152*
Serum type			
Positive	91	72	78
Equivocal	10	18	11
Negative	36	60	63
Standard sera	2nd International Reference Preparation	Working standard, RKI 5/96	International Reference Preparation*
Units	5000 mIU/ml	1000 arbitrary units/ml	

* Dilution series of the International Standard in PBS and negative serum included (12 samples).

Table 2. *Measles, mumps and rubella enzyme immunoassays (EIA) used in the participating countries (all assays ran with internal controls calibrated against the international standard sera for measles and rubella and the German standard sera for mumps)*

	Antigen		
	Measles	Mumps	Rubella
Australia (AU)	Behring*	Behring*	Behring*
Denmark (DK)	Behring*	Behring*	Behring*
Finland (FI)	Behring*	In-house [7]	Behring*
France (FR)	Behring*	Behring*	Behring*
Germany (DE)	Behring*	Behring*	Behring*
Italy (IT)	Behring*	Behring*	Behring*
Netherlands (NL)	In-house	In-house [8, 9]	In-house
Sweden (SE)	In-house	In-house	In-house
United Kingdom (UK)	Gull	Biostat	Microgen

* Enzygnost®.

[7–9]. This in-house standard was calibrated against the international standard for measles and rubella or the working standard for mumps. All original quantitative results were designated as 'local units'. The cut-offs were either given by the kit producer or developed locally (Table 3).

Repeat panels

Each reference laboratory tested their panel four times; twice at the beginning and twice when halfway through testing the main serum bank. All participating countries were requested to test the panel twice. The first round of testing took place before that of the main serum bank to evaluate the sensitivity and specificity of the assays used in each laboratory in comparison to the reference centre before testing the main body of sera, in order to identify any assays which were unsuitable for testing the main body of sera. The second round of panel testing occurred halfway through the main testing. These results were then compared to those from the first round to detect assay drift. The results of panel testing were sent to CDSC, London in an electronic format either by e-mail or on diskette.

For the laboratories testing the panels twice, the

Table 3(*a*). *Cut-off values of the Measles panel* (*values in mIU/ml*) *of the EIAs used in the reference laboratory and in the participating countries. Comparison of the local non-standardized* (*Nstd*) *cut-off with the reference laboratory cut-off expressed in the local units* (*Std*)

		Reference laboratory: Denmark (Behring)		
Country		Negative < 150	Equivocal 150–350	Positive > 350
Australia	Nstd	< 150	150–345	> 345
(Behring)	Std	< 171	171–398	> 398
Germany	Nstd	< 150	150–300	> 300
(Behring)	Std	< 152	152–357	> 357
Finland	Nstd	< 150	150–300	> 300
(Behring)	Std	< 149	149–293	> 293
France	Nstd	< 150	150–300	> 300
(Behring)	Std	< 193	193–488	> 488
Italy	Nstd	< 150	150–300	> 300
(Behring)	Std	< 155	155–356	> 356
The Netherlands	Nstd	< 190	190–300	> 300
(in-house)	Std	< 149	149–336	> 336
UK	Nstd	< 50	50–100	> 100
(Gull)	Std	< 75	75–153	> 153

Table 3(*b*). *Cut-off values of the Mumps panel* (*values in titres* (#) *and arbitrary units* (*). *Titres were used in the regression analysis*) *of the EIAs used in the reference laboratory and in the participating countries. Comparison of the local non-standardized* (*Nstd*) *cut-off with the reference laboratory cut-off expressed in the local units* (*Std*)

		Reference laboratory: Germany (Behring)		
Country		Negative < 230# < 8*	Equivocal 230–500# 8–14*	Positive > 500# > 14*
Australia	Nstd#	<230	230–485	> 485
(Behring)	Std#	< 294	294–710	> 710
Denmark	Nstd#	< 250	250–450	> 450
(Behring)	Std#	< 251	251–500	> 500
Finland	Nstd*	< 10	10–15	> 15
(in-house)	Std*	< 13	13–18	> 18
France	Nstd#	< 230	230–500	> 500
(Behring)	Std#	< 192	192–377	> 377
Italy	Nstd*	< 10	–	> 10
(Behring)	Std*	< 15	15–23	> 23
The Netherlands	Nstd#	< 45	45–60	> 60
(in-house)	Std#	< 50	50–72	> 72
UK	Nstd*	< 8	8–14	> 14
(biostat)	Std*	< 15	15–28	> 28

paired results were compared by plotting the logarithms (base 10) of the titres and drawing the slope through the origin. In general the second test results were used for the standardization, because they were tested at a time closer to main serum bank testing. In some cases, the number of samples available for repeat testing was much reduced, in which case the first test results were used. The first panel was used on

Table 3(*c*). *Cut-off values of the Rubella panel* (*values in IU/ml*) *of the EIAs used in the reference laboratory and in the participating countries. Comparison of the local non-standardized* (*Nstd*) *cut-off with reference laboratory cut-off expressed in the local units* (*Std*)

		Reference laboratory: UK (Microgen)		
Country		Negative < 5	Equivocal 5–10	Positive > 10
Australia	Nstd	< 4	4–7	> 7
(Behring)	Std	< 4·8	4·8–8·9	> 8·9
Germany	Nstd	< 4	4–7	> 7
(Behring)	Std	< 7·9	7·9–18·2	> 18·2
Denmark	Nstd	< 15	–	> 15
(Behring)	Std	< 7·2	7·2–15·4	> 15·4
Finland	Nstd	< 4	4–10	> 10
(Behring)	Std	< 6·3	6·3–13·3	> 13·3
France	Nstd	< 4	–	> 4
(Behring)	Std	< 5·4	5·4–10·6	> 10·6
Italy	Nstd	< 4	4–7	> 7
(Behring)	Std	< 6·9	6·9–15·9	> 15·9
The Netherlands	Nstd	< 10	10–20	> 20
(in-house)	Std	< 7·8	7·8–15·1	> 15·1

three occasions: the Danish measles panel (due to missing values), the Italian mumps (due to a better fit) and the British rubella (due to missing values).

Danish mumps panel (subset *vs.* German)

The Danish test of the mumps panel could not be used for the standardization process. This was related to a change in mumps assay method in Denmark between the first and second rounds of testing. For the first round of testing, an in-house assay was used, whereas a Behring assay was introduced for testing the panel a second time and for the main serological survey. These later panel results could not be used due to very poor regression against the reference laboratory. To enable standardization against Germany, a subset of the Danish main panel (sdm) was sent to the reference laboratory for testing (50 positive, 32 equivocal, 50 negative by Danish results). The Germans also retested their own standardization panel and the Danish standardization panel.

Finnish measles panel (subset *vs.* German)

The measles panel distributed to Finland arrived partially dried out. After rediluting specimens to the original volumes, the test results regressed very poorly against the reference laboratory. To enable standardization, a subset of the Finnish main panel (sfm) was also sent to the German reference laboratory for testing (50 positive, 50 equivocal and 50 negative by the Finnish results). Although Germany was not the reference the German measles results were very close to those of the reference (Danish) results.

Regression analyses

The panel results for measles, mumps and rubella were related to the results of the reference laboratories using regression. Sera with concentrations outside the detection limits were assigned imputed titres. For concentrations below the detection level, the imputed value was half the lower detection level. For concentrations above the detection level, the imputed value was twice the detection level.

Titres were converted to $\log_{10}$ prior to analysis and distributions of log-titres and scatterplots were produced. For each antigen, the log-titres of each laboratory were regressed against those of the reference laboratory. Both linear and quadratic regressions were used. Values of R^2 (the square of the multiple correlation coefficient) were calculated to quantify the proportion of the variation between the testing and reference laboratory accounted for by the

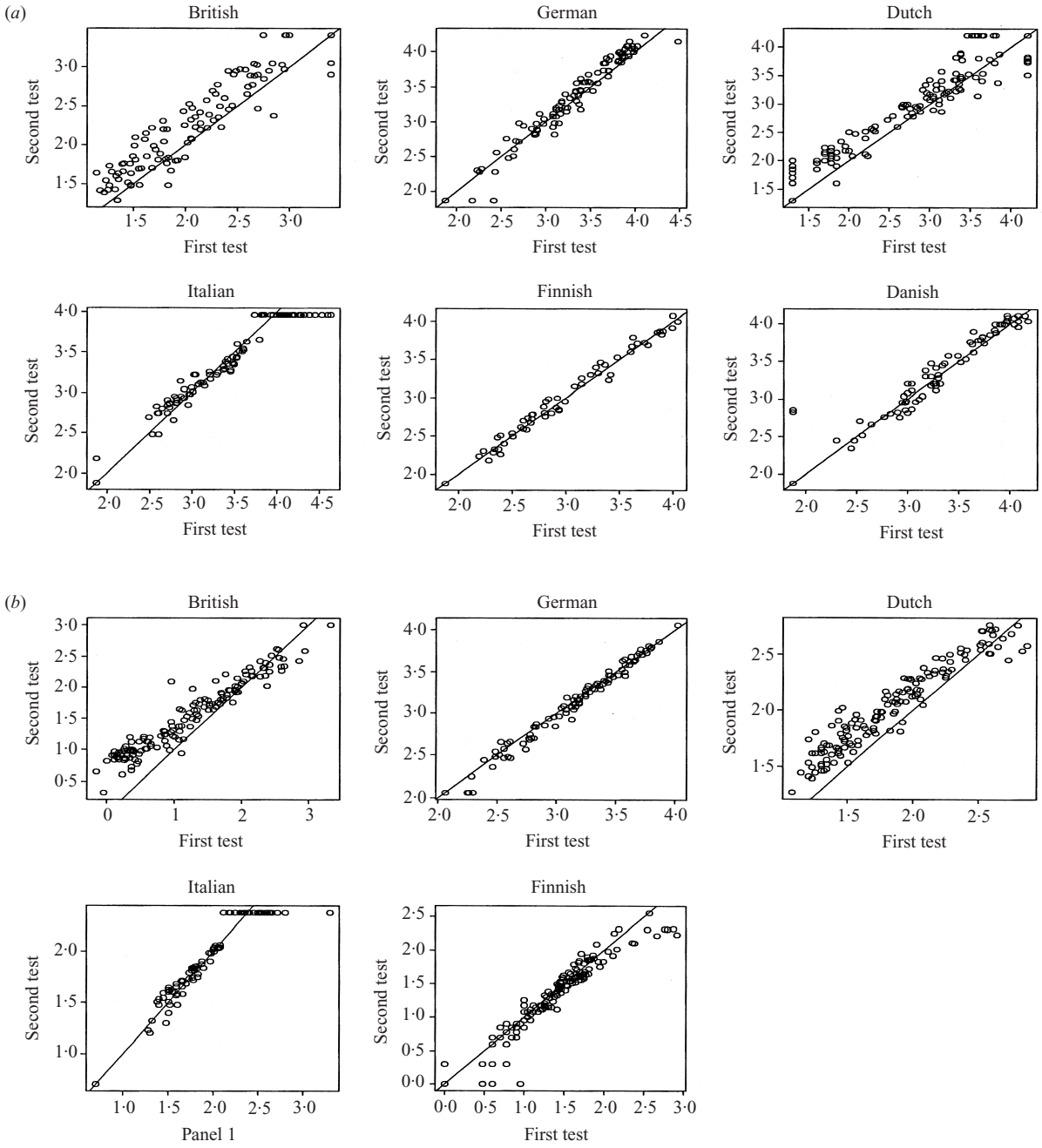

Fig. 1. For legend see facing page.

regression. The best fitting line was selected on the basis of the *F* test. From each regression procedure, a standardization equation was produced to enable the conversion of panel results from local unitage to the standardized reference laboratory unitage.

Quantitative comparison of standardized results

To assess the extent of quantitative agreement with the reference laboratory, the standardized assay results of each country were plotted against those of the reference laboratory.

Qualitative comparison of standardized results

To assess the extent of qualitative agreement with the reference laboratory, the standardized quantitative assay results from each country were classified as negative, equivocal or positive with the same cut-off values used in the reference laboratory. These results were compared with the qualitative results of panel

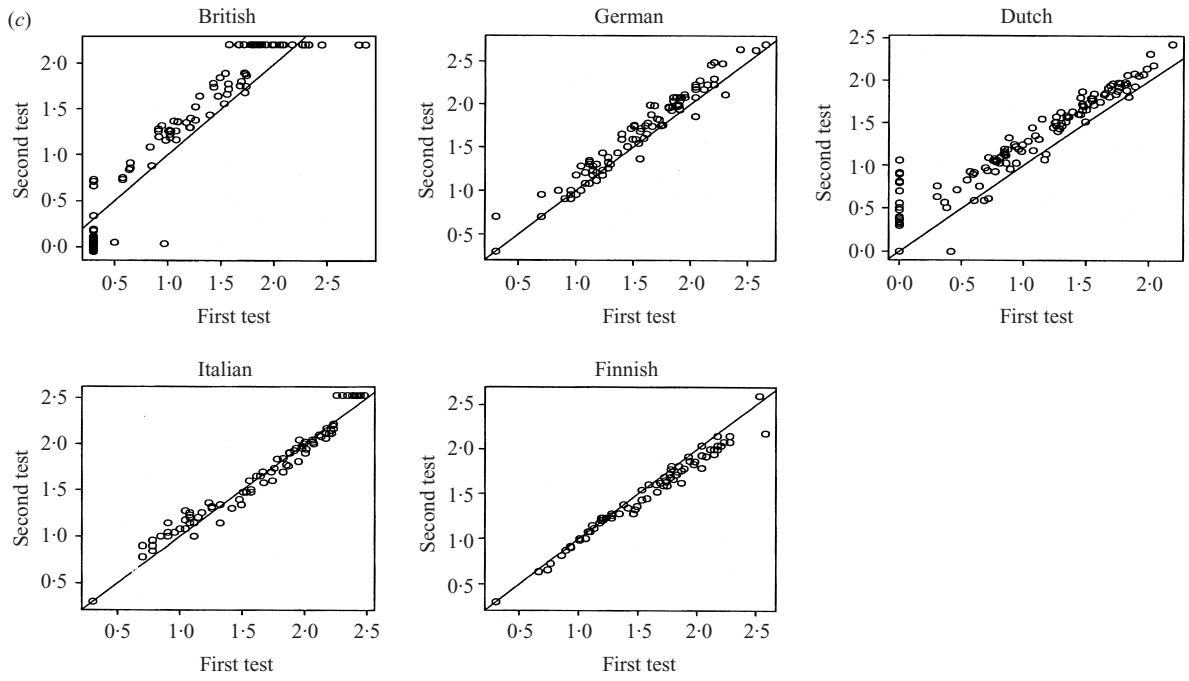

Fig. 1. Pairwise comparison of results of first and second rounds of panel testing: (*a*) Measles panel; (*b*) Mumps panel; (*c*) Rubella panel.

testing in the reference laboratory. For each country, the results were also compared to the qualitative results obtained using the local quantitative value classified with the local cut-off.

Comparison with dilutions of standard sera in negative serum and PBS

A comparison of a dilution series of standard sera was undertaken for the rubella panel. A single, blinded dilution series of the standard sera in negative serum and PBS was included on the panel of sera sent for testing (except to Finland).

RESULTS

The results are summarized in the attached tables and figures.

Repeat panel testing

All countries with two exceptions undertook repeat panel testing. Sweden and Australia only received enough sera to test each panel once.

The measles panel (Fig. 1*a*) tested in Germany, Italy and Finland showed good agreement between the results of the first and second tests. The Dutch and British results were systematically higher on the second panel. In all cases, but one (Denmark), the second panel results were used as testing was closer to that of the main sero-survey results. The Danish second panel agreed well with the first, but had many missing values due to inadequate volumes. The second French panel had many missing sera.

Comparison of the two rounds of mumps panel testing (Fig. 1*b*), showed some upward drift for the Dutch and British panels; and a slight downward drift for the German and Finnish panels. In all cases except one (Italy), the second panel results were used because of closeness to main serum bank testing. For the Italian mumps results the first panel results were used, as there was better fit in the regression.

For the rubella panel (Fig. 1*c*), there was little assay drift overall between the two rounds of panel testing, except for the German, Dutch and United Kingdom panels. For the former two, there was some upward drift. Due to inconsistent results and some missing values for the second British panel test, the original British panel results were used as the reference. The second British test was thus standardized against the first. Otherwise for all countries the second panel results were used.

Pair-wise comparison and regression plots

An example of the distributions, scatterplots and regression lines for each laboratory relative to the reference laboratory are shown in Figure 2(*a*, *b*) together with their standardization equation. As an

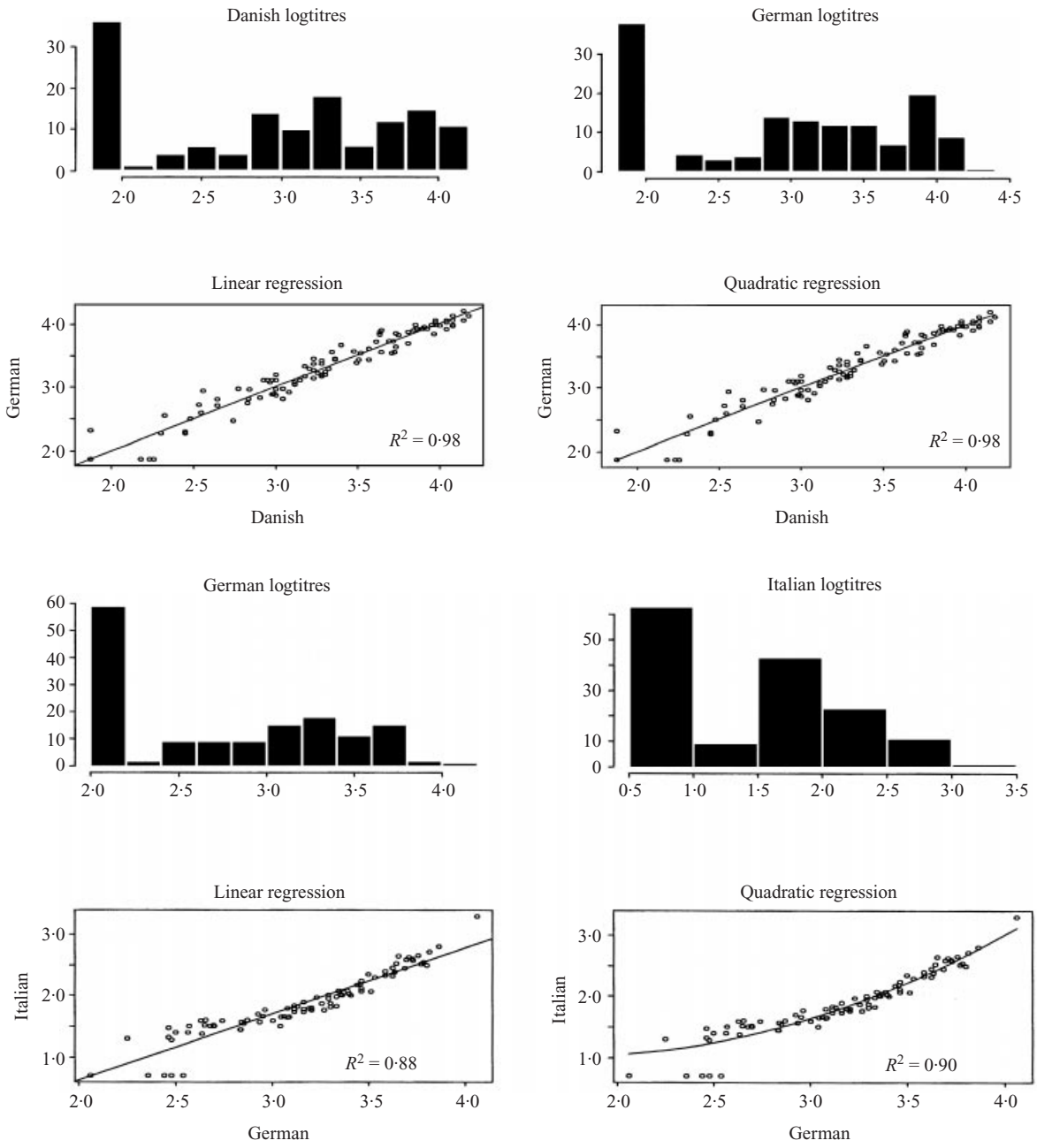

Fig. 2(*a*). Measles panel. Pairwise comparison between the results of the reference laboratory (Denmark) and those of the German laboratory showing the distribution of $\log_{10}$ antibody values, scatter plots, linear and quadratic regressions with R^2 values. Standardization equation: log 10 (German) = $-0{\cdot}02 + 1{\cdot}012$ *log 10 (Danish). (*b*) Mumps panel. Pairwise comparison between the results of the reference laboratory (Germany) and those of the Italian laboratory showing the distribution of $\log_{10}$ antibody values, scatter plots, linear and quadratic regressions with R^2 values. Standardization equation: log 10 (Italian) = 2·192–1·35 *log 10 (German) +0·3885 *log 10 (German)^2.

example of linear regression, the pair-wise comparison between the Danish reference results and the German results for the measles panel is shown (Fig. 2*a*). There is excellent fit with linear regression ($R^2 = 0{\cdot}98$), and no improvement with quadratic regression ($R^2 = 0{\cdot}98$), consequently the linear standardization equation was chosen. To convert 300 mIU/ml according to the German assay into the corresponding unitage of the Danish reference assay: the logarithm of the German reading is first taken [log(300) = 2·48]. The standardization equation is then inverted: $(2{\cdot}48 + 0{\cdot}02)/1{\cdot}012 = 2{\cdot}47$ to give the log of the unitage in Danish reference units. Taking antilogarithms gives the unitage as $10^{(2{\cdot}47)} = 293$. Thus, 300 mIU/ml in

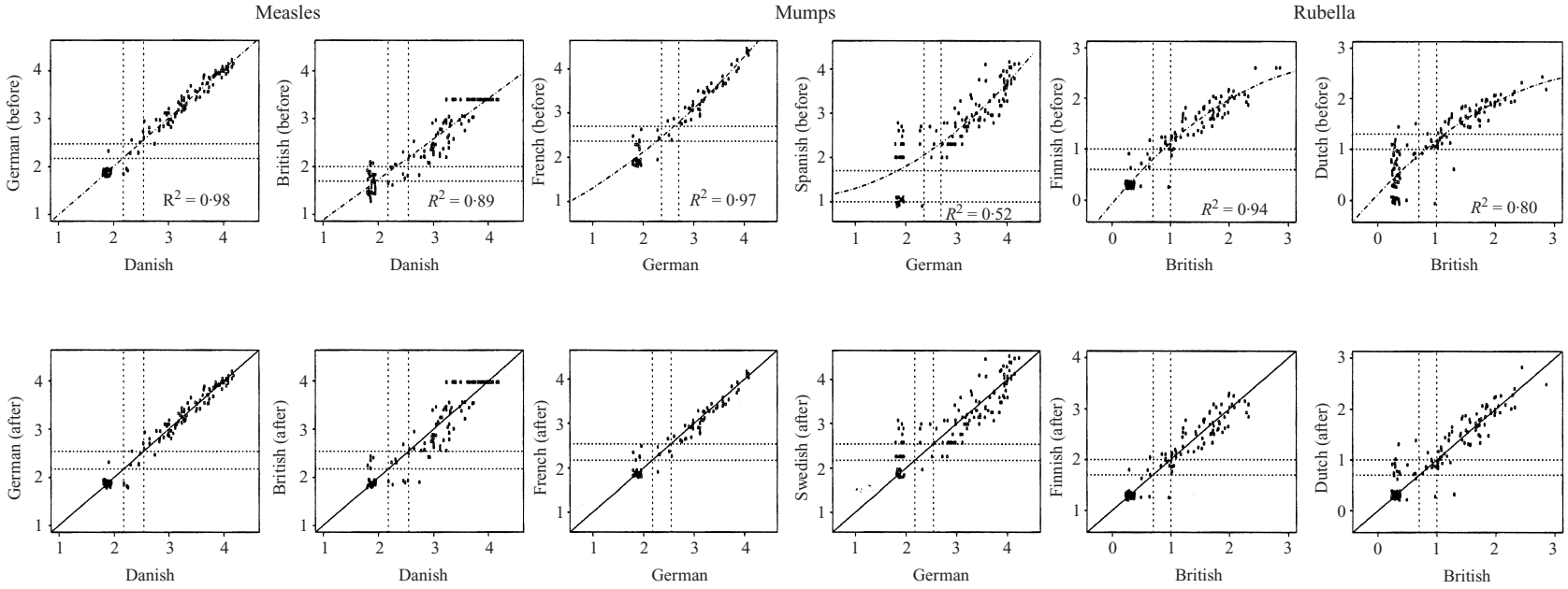

Fig. 3. Log_{10} scatter plots of the participating countries' panel results before and after standardization against the reference laboratory. The plots before standardization show unstandardized results with local cut-offs, R^2 values and regression line used for standardization. The plots after standardization show standardized results (obtained by inverting the standardization equation) with the reference cut-offs and the line of equivalence.

Table 4. *Values of each R^2 for each country and antigen*

	Measles		Mumps		Rubella	
Country	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
Britain	0·89	0·89	0·89	0·89	0·91	0·94
Denmark	Ref	Ref	0·92	0·92	0·93	0·94
Finland	0·95	0·95	0·43	0·45	0·91	0·92
Germany	0·98	0·98	0·96	0·97	0·92	0·94
Italy	0·97	0·97	0·88	0·90	0·92	0·94
The Netherlands	0·83	0·83	0·85	0·86	0·79	0·80
Sweden	0·77	0·77	0·50	0·52	0·78	0·78
Australia	0·95	0·95	0·95	0·95	0·72	0·72

Table 5(*a*). *Qualitative results of measles panel testing by the participating countries – local non-standardized and standardized results according to the reference laboratories results.* (*Non-standardized results are given in parentheses*)

		Reference laboratory: Denmark*		
Country	Qualitative	Negative (N) ($n = 36$)	Equivocal (E) ($n = 10$)	Positive (P)† ($n = 91$)
Australia	N	30 (29)	1 (1)	0 (0)
	E	1 (2)	7 (7)	1 (1)
	P	2 (2)	1 (1)	84 (84)
Finland (subset *vs.* German)	N	49 (50)	0 (0)	1 (1)
	E	13 (12)	16 (21)	7 (17)
	P	0 (0)	5 (0)	59 (49)
France	N	24 (21)	1 (1)	0 (0)
	E	3 (5)	7 (4)	0 (0)
	P	0 (1)	2 (5)	49 (49)
Germany	N	35 (35)	3 (3)	0 (0)
	E	1 (1)	4 (3)	1 (1)
	P	0 (0)	3 (4)	90 (90)
UK	N	31 (18)	5 (2)	1 (0)
	E	5 (15)	4 (5)	2 (1)
	P	0 (3)	1 (3)	88 (90)
Italy	N	35 (34)	2 (2)	0 (0)
	E	0 (1)	2 (2)	0 (0)
	P	0 (0)	3 (3)	91 (91)
The Netherlands	N	30 (35)	4 (5)	2 (2)
	E	6 (1)	3 (1)	3 (1)
	P	0 (0)	3 (4)	86 (88)

* Some countries did not test the whole panel. Missing numbers are as follows. Denmark (Neg, Equiv, Pos): France (9, 0, 42), Italy (1, 3, 0), Australia (3, 1, 6).

† Negative is < 150 mIU/ml, equivocal is 150–350 mIU/ml and positive is > 350 mIU/ml.

German units corresponded to 293 mIU/ml Danish reference units.

Similarly as an example of quadratic regression, the pair-wise comparison between the German reference results and the Italian results for the mumps panel is shown (Fig. 2*b*). The fit improved with the quadratic

Table 5(*b*). *Qualitative results of mumps panel testing by the participating countries – local non-standardized and standardized results according to the reference laboratory results.* (*Non-standardization results are given in parentheses*)

		Reference laboratory: Germany*		
Country	Qualitative	Negative (N) ($n = 60$)	Equivocal (E) ($n = 18$)	Positive (P)† ($n = 72$)
Australia	N	54 (53)	4 (0)	0 (0)
	E	5 (5)	12 (13)	1 (0)
	P	0 (1)	2 (5)	61 (62)
Denmark (subset *vs.* German)	N	38 (38)	3 (1)	12 (11)
	E	3 (3)	18 (20)	8 (9)
	P	0 (0)	1 (1)	49 (49)
Finland	N	29 (24)	4 (4)	2 (1)
	E	5 (10)	5 (3)	7 (7)
	P	18 (18)	9 (11)	62 (63)
France	N	59 (59)	4 (4)	0 (0)
	E	1 (1)	9 (12)	0 (2)
	P	0 (0)	5 (2)	71 (69)
UK	N	52 (12)	2 (0)	0 (0)
	E	7 (38)	10 (0)	1 (0)
	P	1 (10)	6 (18)	71 (72)
Italy	N	59 (59)	4 (4)	0 (0)
	E	1 (—)	2 (—)	0 (—)
	P	0 (1)	12 (14)	72 (72)
The Netherlands	N	43 (39)	6 (4)	0 (0)
	E	14 (12)	7 (6)	2 (0)
	P	3 (9)	5 (8)	70 (72)

* Some countries did not test the whole panel. Missing numbers are as follows. Germany (Neg, Equiv, Pos): Finland (8, 0, 1), France (0, 0, 1), Australia (1, 0, 10).

† Negative is < 230 titre, equivocal is 230–500 titre and positive is > 500 titre.

regression ($R^2 = 0{\cdot}90$) compared to the linear regression ($R^2 = 0{\cdot}88$). The quadratic standardization equation was selected.

For the measles analysis, the regressions against the Danish reference results generally proceeded well. There was one notable outlier in the comparison between the Dutch and the Danish results. This point was not influential and was thus not omitted. There was one outlier when standardizing the subset of the Finnish main panel against Germany, which was omitted.

For the mumps analysis, the relationships were generally less well defined and several outlying, influential points had to be omitted from the regressions. For the Danish standardization, using results from the Danish main panel, it was necessary to omit a number of outliers. For the Italian analysis, all sera for which both results were below the lower detection limit were excluded, as their inclusion distorted the regression line at higher concentrations.

For the rubella analysis, most of the regressions were unproblematic. However, in the regression between Sweden and Britain, one influential outlier had to be omitted.

Comparisons of quantitative standardized results

The $\log_{10}$ plots of a selection of unstandardized and standardized results for the measles, mumps and rubella panels are shown in Figure 3. The remaining plots are available from the authors on request. However, the R^2 value indicating the degree of

Table 5(*c*). *Qualitative results of the rubella panel testing by the participating countries – local non-standardized and standardized results according to the reference laboratory results. (Non-standardized results are given in parentheses)*

Country	Qualitative	Reference laboratory: UK* Negative (N) ($n = 63$)	Equivocal (E) ($n = 11$)	Positive (P)† ($n = 78$)
Australia	N	58 (57)	1 (1)	8 (8)
	E	4 (3)	3 (3)	4 (4)
	P	0 (2)	7 (7)	65 (65)
Denmark	N	59 (61)	1 (9)	0 (9)
	E	3 (—)	9 (—)	13 (—)
	P	0 (1)	1 (2)	64 (68)
Finland*	N	52 (49)	1 (1)	0 (0)
	E	3 (6)	4 (2)	4 (3)
	P	1 (1)	3 (5)	63 (64)
France	N	58 (54)	5 (1)	1 (0)
	E	2 (—)	2 (—)	5 (—)
	P	1 (7)	2 (8)	65 (71)
Germany	N	55 (50)	1 (1)	0 (0)
	E	4 (5)	6 (0)	10 (0)
	P	1 (5)	4 (10)	67 (77)
Italy	N	55 (54)	1 (1)	0 (0)
	E	4 (—)	6 (—)	9 (—)
	P	0 (5)	4 (10)	66 (75)
The Netherlands	N	50 (55)	1 (2)	1 (1)
	E	10 (6)	8 (8)	9 (14)
	P	3 (2)	2 (1)	67 (62)

* Some countries did not test the whole panel. Missing numbers are as follows. UK (Neg, Equiv, Pos): Australia (1, 0, 1); Denmark (1, 0, 1); Finland (7, 3, 11); France (2, 2, 7); Germany (3, 0, 1); Italy (4, 0, 3); The Netherlands (0, 0, 1).
† Negative is < 5 IU/ml, equivocal is 5–10 IU/ml and positive is > 10 IU/ml.

agreement for each country and antigen is shown in Table 4. The agreement was good overall. However, the Swedish mumps and measles regression ($R^2 = 0{\cdot}50$ and 0·77 respectively) and the Finnish mumps ($R^2 = 0{\cdot}43$) show large differences from the reference.

Comparisons of qualitative standardized results

Using the cut-offs from the reference laboratories, the standardized, qualitative results for the antibodies against each antigen are presented in Tables 5*a–c*. To examine the qualitative improvement from standardization, comparisons were also made with the reference laboratory using each country's cut-off on the non-standardized results (Tables 5*a–c*).

For the measles panel, no sera were discrepant in more than one country compared to the Danish reference.

For the mumps panel, a number of sera were classified as negative on the German reference panel (Table 5*b*), but positive in Finland (18/52). All (18/52) of these sera remained positive after standardization: standardization thus failing to adjust for local observations. For the panel tested in Britain a large number of negative reference sera were classified as equivocal (38/60) in local units. After standardization, these false equivocals were frequently reclassified as true negative. For the subset of the Danish main serosurvey which was retested in Germany, a large number of sera which tested negative in Denmark, tested positive in Germany (12/53), both before and after standardization.

For the rubella panel, there was generally good

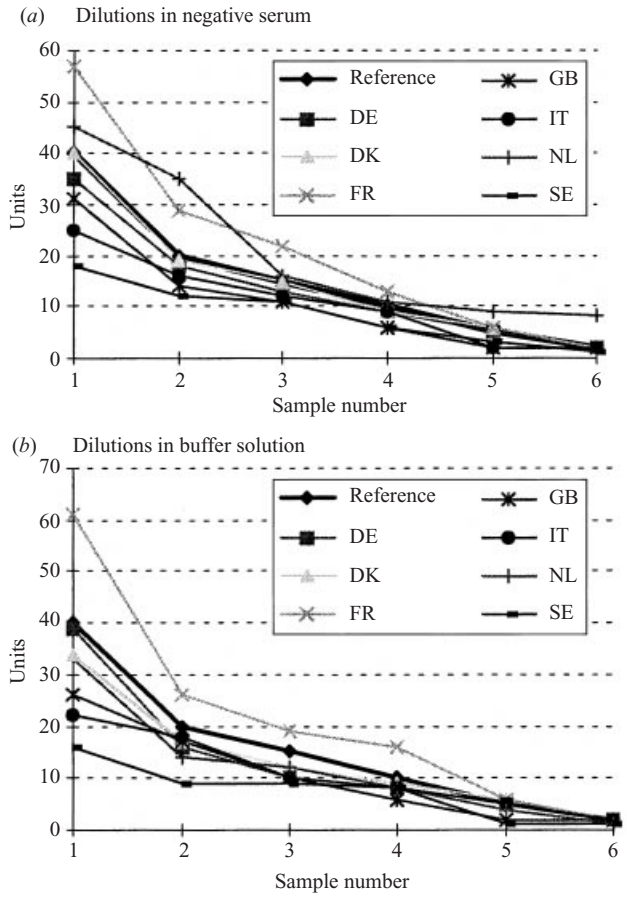

Fig. 4. Different dilution series of the International Standard Serum for rubella included as blind samples in the rubella standard panel and tested by the participating countries (local non-standardized results in IU/ml). (*a*) Dilutions in negative serum; (*b*) dilutions in buffer solution.

agreement with the British reference laboratory (Table 5*c*).

Comparison of dilution series of an International Standard Serum for rubella

The dilution curves for the rubella standard are shown in Figure 4(*a*, *b*). They show substantial differences in local unitages for the different countries using a variety of assays. Six countries used Behring, two in-house and one other commercial assay. For example, 40 mIU/ml of rubella reference sera were reported as 18–57 mIU/ml by different countries. The Danish and German tests gave results closest to the standard dilution values. Dilution in negative serum produced marginally better results than dilution in PBS.

There did not appear to be a correspondence between assay method and closeness to the reference values. For example, The Netherlands using an in-house assay obtained results much closer to those of the reference laboratory than France using a Behring modified EIA assay.

Comparison of cut-off values

The local cut-off value used in the participating countries compared to the reference cut off value 'back-standardized' into local units are shown in Tables 3*a–c*. For example in the measles panel (Table 3*a*), the Danish cut-off standardized to local units (75 mIU/ml) is greater than the local cut-off used in the United Kingdom (50 mIU/ml). The former used the Behring assay, the later the Gull test.

DISCUSSION

ESEN is the first study in which there has been a co-ordinated effort to generate comparable sero-prevalence data for vaccine preventable infections across countries. Interpretation of these seroprevalence data will be used to evaluate the relative effectiveness of the different vaccination programmes in these countries.

Testing with a variety of assay methods (such as haemagglutination inhibition, neutralizing test, enzyme immunoassay) can provide very different results [6]. Even investigating the same sera with a variety of kits based on the same method, can lead to significantly different results [3]. This is despite attempting to achieve comparability using internal controls, measurement correction factors and calibrating against an international standard. To achieve comparability of the serological survey results from different countries, the ideal option would have been to test all sera with the same assay in a single laboratory. It was agreed for the project that participants would continue to use their usual assay method, as they would be familiar with this technique and it would make the project overall more sustainable. If countries changed to a particular assay for the duration of the project, then reverted to their original methods, intra-country comparability would have been lost. Thus within the project, all participants continued to use their current assays based on the EIA method, but with a variety of kits. A standardization procedure had to be developed to allow direct comparison between assay results obtained in different ways. The method involved comparing quantitative

results of antibody concentration, by calibrating each country's EIA units against those of the reference laboratory. This does not involve a gold standard to provide an absolute truth.

There were some problems with the standardization process. Although complete panels of sera were distributed in the majority of instances, towards the end of the project some countries were provided with smaller volumes of sera than others due to a limited supply of reference sera. This meant some participants were unable or only partially able to test the panel a second time. In future, adequate volumes of sera need to be prepared. We were able to overcome this problem by taking a sample of the main serosurvey, which was then retested in a reference centre. These results were then used to obtain a standardization equation.

Some assay drift also occurred. This technique of standardization only adjusts for between-laboratory variability. The standardization equations are derived on the assumption that any observed variation was non-random and constant over time. Variation in results over time (assay drift) was presumably related to in-house assay-to-assay variation and was detected by re-testing the panel. Control of this within laboratory variability was the responsibility of the quality control procedures in each laboratory. Using the standardization equation generated by repeat panel testing during the period of testing the main serum collection should help to adjust for this assay drift. However, to continue to undertake inter-country comparisons with this standardization technique, will require the regular distribution of panels of sera and maintaining internal quality control.

Both the Swedish and Finnish mumps panel regressed poorly against the German reference. Many of the sera classified as negative by the German reference laboratory were classified as positive by Finland when non-standardized. This occurred for both the non-standardized and standardized results – so standardization was unable to reduce this observation. One possible reason for this observation may be the influence of the virus strain used as antigen in the kit. As the vast majority of sera from the main serosurvey results will be seropositive to mumps antibody in this highly vaccinated population, it was not feasible to sample and re-test the main results to identify potential false positives. Hence, the main standardized serosurvey results for Finland may overestimate the prevalence of seropositivity to mumps antibody. In Sweden, a decision has been taken to change the EIA assay method to test the main serosurvey.

Furthermore, for the standardization to work well the test used (particularly that of the reference laboratory) should ideally give a continuous outcome with a detection limit below the negative cut-off (this was often not the case with the Behring test). If the detection limit is the same as the negative cut-off then potentially high negatives could not be standardized upwards. The only example of this was with the French mumps panel where the standardized cut-off of < 192 is below the detection limit of the tests.

International units have been the traditional approach to producing 'comparable' results between laboratories and countries. They are based on international standard sera which exist for measles and rubella. They allow the calibration of titres, optical densities or indices from different laboratories into international units [2]. The lack of a mumps international standard has presented problems for international comparability, which meant that a working standard had to be created for the purposes of this project. The creation of a European or international mumps standard would enable laboratories express mumps antibody concentrations in common units, as for measles and rubella.

Our results also indicate that there still remains a residual lack of comparability between the unitage derived even from calibrating to international standards. The wide variation in the laboratory cut-offs expressed in standardized units has been noted before [3]. We also found a substantial difference in the unitage of the rubella standard dilution series. Some of this variation may lie within normal test variation, but this does not account for all the observed difference. This suggests that direct inter-country comparisons of EIA results based purely on an international standard need to be made cautiously. We now have a working tool for the comparison of MMR EIA results. This will play a key role in comparing the results of the serological surveys collected as part of this project. A further panel of sera containing MMR in one serum is currently under collection. This will enable this method to continue to be used within these ongoing collaborative projects.

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