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Establishment of replacement International Standard 13/132 for human antibodies to *Toxoplasma gondii*

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ABSTRACT

Sixteen laboratories carried out a collaborative study to validate 13/132 as a replacement International Standard (IS) for TOXM (3rd IS for anti-Toxoplasma Serum, Human, 1000 IU). 13/132 is a freeze dried preparation of pooled human plasma from six donors who experienced a recent *Toxoplasma gondii* infection. The potency of 13/132 was compared to TOXM and 01/600 (1st IS for anti-Toxoplasma IgG, Human, 20 IU). Samples were tested for IgA, IgG, IgG avidity and IgM in agglutination assays; enzyme linked immunosorbent assays (ELISA), enzyme linked fluorescent assays, immunoblots, immunofluorescence assays and the Sabin–Feldman dye test for Ig. 13/132 was strongly positive for Ig, IgA, IgG and IgM and the reproducibility was very good. 13/132 contains high levels of anti-Toxoplasma Ig, IgG and IgM and its potency falls between TOXM and 01/600. The avidity of IgG was found to be low, similar to the avidity of IgG from TOXM. 13/132 was established by the Expert Committee on Biological Standardization as the 4th IS for Antibodies, Human, to *T. gondii* with an assigned unitage of 160 IU per ampoule for Ig by dye test and 263 U per ampoule for IgG by ELISA.

1. Introduction

Toxoplasmosis is caused by the parasite *Toxoplasma gondii*. Congenital transmission of *T. gondii* remains a considerable burden on global health, with the highest incidence of 3.4/1000 births reported for South America [1]. The main objective of screening programmes is to prevent infection of the foetus by the parasite during pregnancy, and serology is widely used to diagnose Toxoplasmosis during pregnancy [2]. In addition, toxoplasmosis is a major cause of mortality among transplant patients [3]. The provision of appropriate antibody standards enables diagnostic laboratories and manufacturers of diagnostic tests to validate serologic assays to diagnose this infection. In 1994, Hansen et al. carried out a collaborative study to validate TOXM as a reference reagent for anti-Toxoplasma Ig by Sabin–Feldman dye test. Each ampoule of TOXM was assigned 1000 IU of anti-Toxoplasma Ig and subsequently established as the 3rd International Standard (IS) by the Expert Committee on Biological Standardization (ECBS) of the World Health Organization [4]. The dye test is a complement-mediated cell killing assay, utilising toxoplasma tachyzoites and does not distinguish between immunoglobulin classes that bind complement [5]. Although the assay is now carried out by fewer laboratories, the dye test is still considered a reference test and a confirmatory assay to validate commercial assays [6]. Therefore the dye test remains an important assay for the standardisation of anti-toxoplasma Ig levels in individuals suspected of toxoplasmosis. TOXM is used by manufacturers of *in vitro* diagnostic tests, national reference laboratories and hospital laboratories. Since 2000, stocks of TOXM have been low and these are now nearly exhausted. In 2003, 01/600 was established by ECBS as the 1st IS for anti-Toxoplasma IgG with a unitage of 20 IU per ampoule relative to TOXM [7,8]. 01/600 has a low level of IgG, which falls within the linear range of commercially available

Abbreviations: CV, coefficient of variation; ECBS, Expert Committee on Biological Standardization; ELFA, enzyme linked fluorescent assay; ELISA, enzyme linked immunosorbent assay; GCV, geometric coefficients of variation; GM, geometric mean; HSDA, high sensitivity direct agglutination assay; IFA, immunofluorescence assay; IS, International Standard; ISAGA, imunosorbent agglutination assay; kD, kilo Dalton; NIBSC, National Institute for Biological Standards and Control.

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Table 1
Characterisation of samples used in this study.

Study code	NIBSC code	Description of source material	Results of native material		Results of freeze dried material	
			Dye test[a] (IU mL^{-1})	IgM ELISA[b] (EU mL^{-1})	Dye test (IU mL^{-1})	IgM ELISA (EU mL^{-1})
TOXM	TOXM	3rd IS for anti-Toxoplasma Ig Human	–	–	1000[c]	–
A	01/600	1st IS for anti-Toxoplasma IgG Human	–	–	20[c]	–
B	01/576	Pool of seven normal human sera	–	–	<2[c]	–
C and E	13/132	Candidate IS for antibodies, human, to *T. gondii* from a pool of 6 plasma donations[d]	794 (500–1000)	101 ± 12	561 (500–1000)[ns]	98 ± 7[ns]
D	174	Anti-Toxoplasma plasma from one donor[e]	1000	105 ± 1	1000	125 ± 7
F	637	Anti-Toxoplasma plasma from one donor[e]	250	94 ± 6	500	101 ± 7

[ns]: Differences between native and freeze dried samples are not significant.
–: Not done.
[a] Lab code 6.1. Results given as geometric mean titre (range).
[b] Lab code 6.2. Results given as geometric mean ELISA Unit (EU) ± standard deviation: > 40: positive; >100: strongly positive (12).
[c] Taken from Rigsby et al., 2004 [8].
[d] Taken from 6 measurements over three days.
[e] Taken from 2 measurements over two days.

immunoassays used by diagnostic laboratories to distinguish between historic, background and diagnostic levels of IgG. Although, the unitage of 01/600 can be traced back to the 2nd IS TOXS, ECBS did not consider IS 01/600 a suitable replacement for TOXM because of the low levels of specific IgG and absence of specific IgM. The committee decided that a replacement for TOXM should contain high levels of IgM and IgG [4,7,8].

Recently, plasma samples from acute cases of Toxoplasmosis were acquired and a preliminary analysis showed that individual samples and the pooled sample 13/132, contained high levels of specific IgM and IgG, the latter with low to borderline avidity (see Table 1 and results not shown). Specific IgG of high avidity is seen as a marker of latent toxoplasmosis, whereas IgG of low avidity can be indicative of a recent infection [9,10]. A collaborative study was designed to validate 13/132 as an IS to replace TOXM. Participants were asked to test 13/132 in the dye test, and in addition were encouraged to use assays that are part of their diagnostic routine. The primary aims of the study were to:

1) assess the suitability of 13/132 as an IS for human anti-Toxoplasma Ig.
2) compare the reactivity of 13/132 relative to TOXM and 01/600 in the dye test.
3) compare the reactivity of 13/132 relative to TOXM and 01/600 in immunoassays for IgM and IgG, including avidity assays.
4) assess the reactivity of 13/132 in agglutination assays, immunoassays and in other titration assays currently in use.

2. Materials and methods

2.1. Participating laboratories and assay codification

Sixteen laboratories from 12 countries, including national reference laboratories, took part in the collaborative study. Details are given in the acknowledgement section. Throughout the study, participating laboratories were identified by a randomly assigned code number to maintain confidentiality. Data were collected and analysed at the National Institute for Biological Standards and Control (NIBSC). Each participant received two sets of seven samples comprising coded ampoules A to F including 01/600 (A) and duplicates of 13/132 (C and E), and one ampoule of TOXM (see Table 1).

2.2. Samples used in the study

Samples labelled A to F and TOXM were distributed as lyophilised preparations in duplicate sample packs by courier at room temperature. The samples were reconstituted following 'instructions for use' issued by NIBSC. Samples of 13/132 that were exposed to an elevated temperature range (−20 °C to +45 °C) to ascertain stability of the active component were distributed on dry ice. A brief characterisation of the samples, study codes, NIBSC codes and their reactivity in the dye test and the IgM capture enzyme linked immunosorbent assay (ELISA) are given in Table 1.

2.3. Characterisation of the proposed International Standard 13/132

Plasma samples were donated with informed consent by 6 female individuals of 21–33 years of age and obtained from Cerba Specimen Services (Saint-Ouen l'Aumône, France). At NIBSC, all samples tested negative for antibodies to Human Immunodeficiency Virus 1 and 2, Hepatitis C RNA and Hepatitis B surface antigen. Samples were stored at −80 °C until further use. Prior to pooling, samples were defrosted and stored at 2–8 °C overnight. The next day, samples were pooled (volume appr. 3 L) during which clotting occurred. Clots were removed by a filtration step using Whatman filter paper (1001-150). The filtrate of the pool was stored at 2–8 °C overnight and dispensed in 0.5 mL aliquots into glass ampoules coded 13/132 on the following day. The mean fill weight for 123 ampoules was 0.5156 g (CV of 0.16%). On the same day, freeze-drying under vacuum was started and completed after four days. Ampoules were back filled with pure N_2 and the mean O_2 content of 12 ampoules was 0.17% (CV of 53.71%). This implies ampoules passed the test for integrity, because the presence of cracks would be associated with an O_2 level of 21% similar to that found in the atmosphere. The mean residual moisture level in 12 ampoules was 0.6608% (CV of 18.89%). One hundred and sixty ampoules were rejected during the production process, 50 ampoules were held for accelerated degradation studies and 3695 ampoules were stored at −20 °C. These are available for distribution by NIBSC.

Native and freeze-dried samples of 13/132 and individual samples 174 (D) and 637 (F), which are part of the serum pool, were tested by dye test and IgM capture ELISA to determine the effect of freeze drying on specific Ig and IgM respectively (see Table 1). No significant differences in the mean values for levels of specific IgM and Ig were found before and after freeze-drying. Differences in unitage determined by dye test were found following freeze drying for samples C and E (13/132) and samples D and F. These fall within the four fold range and are therefore not considered significant.

2.4. Diagnostic assays

An overview of the 24 assay formats used for the detection of anti-*T. gondii* antibodies and their laboratory code is given in Table 2. Titration methods were represented by seven assay formats. Five assays were developed in-house: the dye test for Ig, the high sensitivity direct agglutination assay (HSDA) for IgG [11], immunofluorescence assays (IFA) for IgG and IgM, and the imunosorbent agglutination assay (ISAGA) for IgA and IgM [12]; the Toxoreagent kit for IgG/IgM (Mast) and the ISAGA for IgM (bioMérieux) are commercially available.

Six ELISA and enzyme linked fluorescent assay (ELFA) formats were used to detect IgM; four assays are commercially available (Abbot, bioMérieux, Biorad and Diasorin) and two capture ELISAs were developed in-house [13,14]. Eight ELISAs and ELFAs, including avidity assays, were used to detect specific IgG: five of these are commercially available (Abbot, bioMérieux, Diasorin) and three ELISAs were developed in-house. Immunoblots were used for the detection of IgG (LDBio Diagnostics) and IgM [15]. One competition ELFA (bioMérieux) was used to detect Ig.

2.5. Data analysis

Samples were tested in duplicate on two different days. Data sets containing raw data, transformed data and operating procedures were submitted to NIBSC for analysis. For the majority of methods, reported results (endpoint titres, potencies in IU or ELISA Units [EU] etc.) were converted directly into relative potencies by dividing by the result obtained for the appropriate standard. For IgM data, relative results (given as index, signal/cut off ratio etc) are shown for ELFAs and ELISAs and these cannot be directly interpreted as relative potency. ELISA data from lab 3 were analysed by parallel line bioassay comparing assay response to log concentration in a four-parameter logistic model using version 5.0 of EDQM's CombiStats software [16]. The final estimate in each assay for 13/132 was taken as the geometric mean (GM) of the two coded duplicates (C and E). All mean estimates shown in this report are unweighted GM estimates. Variability between laboratories has been expressed using geometric coefficients of variation (GCV) = $\{10^s - 1\} \times 100\%$ where s is the standard deviation of the $\log_{10}$-transformed estimates.

3. Results and discussion

3.1. Titration assays

Titration assays were carried out by 11 laboratories and all reported results for TOXM and samples A to F. All participants correctly identified sample B as negative. The potencies of the coded positive samples relative to TOXM, 01/600 and 13/132 are summarised in Table 3. Participant 14 did not identify TOXM and 01/600 as positive by dye test and the data of lab code 14.1 were therefore not included in the calculation of the relative potency of 13/132. In addition, data sets which did not contain numerical values for TOXM or 01/600, or which qualified samples A to F as positive or negative were not included in the calculation of the relative potency of 13/132.

Analysis of the dye test and the IFA results for duplicates C and E (13/132) showed that the potency of C relative to E fell within a two-fold difference relative to 1 (range 0.5–2.0) in all laboratories but one, which indicates adequate diagnostic precision among participating laboratories. Commercial and in-house agglutination assays performed better than the dye test in this respect (see Fig. 1).

Hansen et al. assigned a unitage of 1000 IU per ampoule TOXM for Ig [4]. The potency of anti-Toxoplasma Ig in sample A (01/600) relative to TOXM is reported as 0.02, which is equal to a unitage of 20 IU per ampoule and identical to the unitage assigned to 01/600 in a previous collaborative study [7,8,17]. 13/132 (C and E) had a GM

Table 2
Assays used in this study.

Type of test	Manufacturer and name of the test, antibody specificity (*n*)	Participant code	Lab code
Titration assays (*8*)			
Agglutination assay	In-house, HSDA,[a] IgG (*2*)	5, 9	5.1, 9.1
	bioMérieux ISAGA,[b] IgM (*4*)	4, 5, 7, 11	4.1, 5.2, 7.1, 11.1
	In-house, ISAGA, IgA (*1*)	9	9.2
	In-house, ISAGA, IgM (*2*)	2, 9	2.1, 9.3
	MAST Latex toxoreagent, IgG/IgM (*1*)	4	4.2
Dye test	In-house, Ig (*6*)	1, 2, 3, 6, 10, 14	1.1, 2.2, 3.1, 6.1, 10.1, 14.1
IFA[c]	In-house, IgG (*1*)	7	7.2
	In-house, IgM (*1*)	7	7.3
Enzyme immunoassays and enzyme linked fluorescent assays (*15*)			
ELFA[d]	bioMérieux VIDAS Toxo Competition (*1*)	13	13.1
	bioMérieux VIDAS Toxo IgG II (*9*)	1, 2, 5, 7, 8, 10, 12, 13, 16	1.1, 2.2, 5.3, 7.4, 8.1, 10.2, 12.3, 13.2, 16.1
	bioMérieux VIDAS Toxo IgG Avidity (*1*)	16	16.2
	bioMérieux VIDAS Toxo IgM (*10*)	1, 2, 5, 7, 8, 10, 12, 13, 15, 16	1.2, 2.3, 5.4, 7.5, 8.2, 10.3, 12.4, 13.3, 15.3, 16.3
ELISA[e]	Abbott ARC Toxo IgG (*2*)	11, 12	11.2, 12.1
	Abbott ARC Toxo IgG Avidity (*1*)	11	11.3
	Abbott ARC Toxo IgM (*2*)	11, 12	11.4, 12.2
	Bio Rad Platelia Toxo IgM (*1*)	4	4.3
	Diasorin Liaison Toxo IgG (*1*)	11	11.5
	Diasorin Liaison Toxo IgM II (*1*)	11	11.6
	In-house, IgG (*2*)	4, 15	4.4, 15.1
	In-house, IgG avidity (*1*)	15	15.2
	In-house, IgM capture (*2*)	3, 6	3.2, 6.2
Immunoblot	In-house, IgM (*1*)	4	4.5
	LDBio Diagnostics IgG (*1*)	11	11.7

[a] High sensitivity direct agglutination assay [11].
[b] Imunosorbent agglutination assay [12].
[c] Immunofluorescence assay.
[d] Enzyme linked fluorescent assay.
[e] Enzyme linked immunosorbent assay.

Table 3
Summary of results from titration assays for samples A to F relative to TOXM, 01/600 and 13/132.

Assay (antibody)	Test sample	A (01/600)	C/E (13/132)		D			F		
	Reference	TOXM	TOXM	01/600 (A)	TOXM	01/600 (A)	13/132 (C/E)	TOXM	01/600 (A)	13/132 (C/E)
Dye test (Ig)	Lab code 1.1	0.02	0.54	28.8	0.77	40.7	1.41	0.24	12.8	0.45
	Lab code 2.2	0.03	0.28	8.8	0.50	15.6	1.78	0.25	7.8	0.89
	Lab code 3.1	0.02	0.33	21.1	0.23	14.9	0.71	0.20	12.5	0.59
	Lab code 6.1	0.02	0.32	19.7	0.63	39.4	2.00	0.25	15.6	0.79
	Lab code 10.1	0.03	0.21	6.7	0.35	11.3	1.68	0.31	9.8	1.46
	GM[a]	0.02	0.32	14.78	0.46	21.15	1.43	0.25	11.40	0.77
	GCV[b]	42.7%	41.0%	85.9%	60.7%	81.4%	51.2%	17.3%	30.7%	56.1%
Latex MAST (IgM, IgG)	Lab code 4.2	0.55	0.42	15.4	0.50	18.3	1.19	0.35	13.0	0.84
IFA (IgM)	Lab code 7.3	Not done	0.50	Not done	0.42	Not done	0.84	0.42	Not done	0.84
ISAGA (IgM)	Lab code 2.1	Not done	0.26	Not done	0.26	Not done	1.00	0.26	Not done	1.00
IFA (IgG)	Lab code 7.2	0.01	0.42	67.3	0.50	80.0	1.19	0.25	40.0	0.59
HSDA (IgG)	Lab code 5.1	0.06	0.50	8.0	0.50	8.0	1.00	0.50	8.0	1.00
	Lab code 9.1	0.02	0.13	6.7	0.36	19.0	2.83	0.25	13.5	2.00
	GM[a]	0.03	0.25	7.3	0.42	12.3	1.68	0.35	10.4	1.41

[a] GM: geometric mean.
[b] GCV: geometric coefficients of variation.

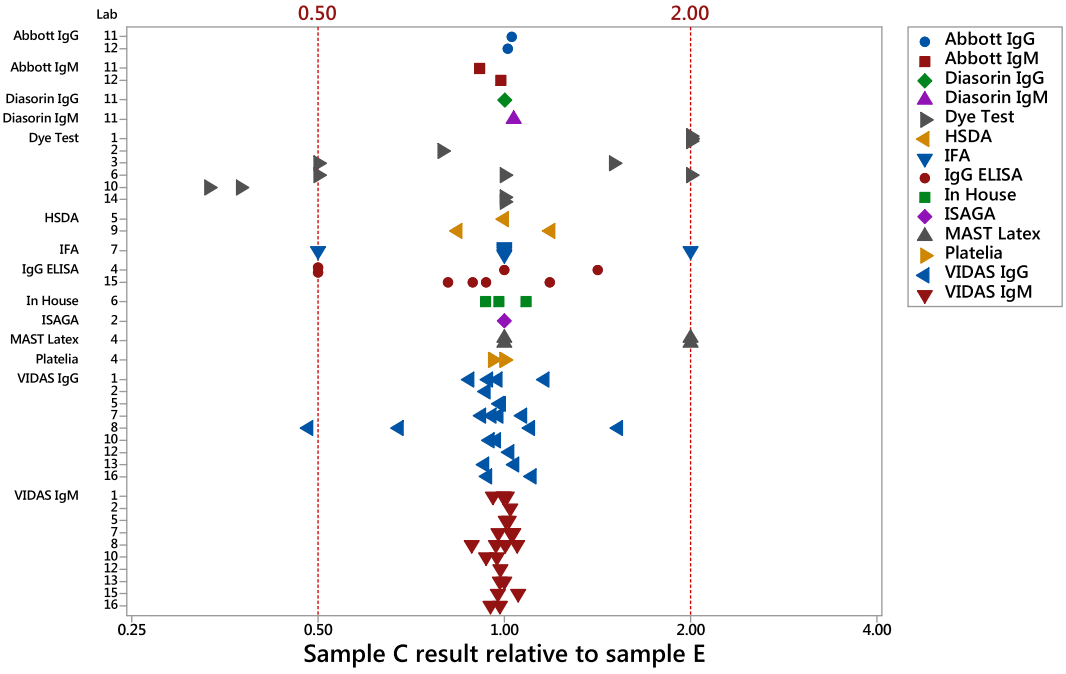

Fig. 1. Relative results of coded duplicate samples C and E (representing 13/132) are presented for various assays. Most ratios fall within the twofold range (50–200%) relative to a C/E ratio of 1. Only participant 10 reported results for the dye test that exceeded this range. In general, the highest precisions were achieved by commercial ELISAs and ELFAs.

potency of 0.32 with a GCV of 41.0% in the dye test relative to TOXM and a potency of 14.78 with a GCV of 85.9% relative to 01/600 (Table 3). The calculated unitage of 13/132 for Ig is therefore 320 IU mL^{-1} or 160 IU per ampoule relative to TOXM and 296 IU mL^{-1} or 148 IU per ampoule relative to 01/600.

The relative potency of 13/132 compared to TOXM varied in other agglutination assays. Compared to the dye test, the Toxoreagent kit (Mast) gave the closest results for samples C and E (13/132), D and F. This assay does not distinguish between IgG and IgM, whereas IFA and ISAGA specifically detect either IgG or IgM. Thus the differences in unitage for the latter two assays may reflect differences in assay procedures and in antibody classes detected.

3.2. Enzyme immunoassays and enzyme linked fluorescent assays

All participants who carried out ELISAs and ELFAs to detect IgM or IgG reported results for TOXM and samples A to F. Sample B was reported as negative for IgM and IgG. The results of quantitative assays, which failed to assign a numerical value to TOXM, were not used to assign a unitage and are excluded from Tables 4 and 5. The results of qualitative assays for IgG and IgM are presented in Table 6.

Sample A (01/600) was reported as negative for IgM by in-house ELISAs but in commercial assays a very low value for IgM relative to TOXM was reported (see Table 4). The results of 13/132 and samples D and F for IgM relative to TOXM, 01/600 and 13/132 are summarised in Table 4. As mentioned above, the low IgM values of 01/600 resulted in artificially high IgM values for 13/132 and samples D and F. These figures should thus be considered for information only.

Table 4
Summary of IgM results in ELISA and ELFA for samples A to F relative to TOXM, 01/600 and 13/132.

Assay	Test sample	A (01/600)	C/E (13/132)		D			F		
	Reference	TOXM	TOXM	01/600 (A)	TOXM	01/600 (A)	13/132 (C/E)	TOXM	01/600 (A)	13/132 (C/E)
bioMérieux VIDAS Toxo IgM	Lab code 1.2	0.01	0.76	53.0	0.72	50.0	0.94	0.85	59.3	1.12
	Lab code 2.3	0.02	0.79	48.7	0.76	46.3	0.95	0.87	53.1	1.09
	Lab code 5.4	0.02	0.77	45.0	0.75	43.6	0.97	0.99	21.5	1.01
	Lab code 7.5	0.02	0.76	49.6	0.73	47.7	0.96	0.87	50.8	1.13
	Lab code 8.2	0.02	0.80	53.9	0.81	52.9	0.98	0.95	63.0	1.17
	Lab code 10.3	0.01	0.69	61.4	0.66	59.8	0.97	0.79	69.3	1.13
	Lab code 12.4	0.01	0.76	51.7	0.75	51.0	0.99	0.84	57.4	1.11
	Lab code 13.3	0.01	0.74	50.8	0.72	49.2	0.97	0.85	58.2	1.15
	Lab code 15.3	0.01	0.74	57.1	0.73	55.1	0.97	0.85	64.6	1.13
	Lab code 16.3	0.01	0.72	49.5	0.72	50.2	1.01	0.80	55.2	1.11
	GM[a]	0.01	0.75	51.9	0.73	50.4	0.97	0.85	58.6	1.13
	GCV[b]	12.3%	4.5%	9.1%	5.0%	9.3%	2.0%	5.1%	9.8%	2.0%
Abbott ARC Toxo IgM	Lab code 11.4	0.01	0.53	79.2	0.45	68.0	0.86	0.67	100.3	1.27
	Lab code 12.2	0.01	0.45	65.6	0.36	52.7	0.80	0.57	82.7	1.26
	GM[a]	0.01	0.49	72.1	0.41	59.9	0.83	0.62	91.1	1.26
Biorad Platelia	Lab code 4.3	0.05	0.98	21.3	0.97	21.0	0.99	0.99	21.5	1.01
IgM capture ELISA	Lab code 3.2	Negative	0.25	Negative	0.20	Negative	1.02	0.25	Negative	0.93
	Lab code 6.2	Negative	0.67	Negative	0.70	Negative	1.04	0.64	Negative	0.95
	GM[a]	–	0.41	–	0.37	–	1.03	0.40	–	0.94

[a] GM: geometric mean.
[b] GCV: geometric coefficients of variation.

Table 5
Summary of IgG results in ELISA and ELFA for samples A to F relative to TOXM, 01/600 and 13/132.

Assay	Test sample	A (01/600)	C/E (13/132)		D			F		
	Reference	TOXM	TOXM	01/600 (A)	TOXM	01/600 (A)	13/132 (C/E)	TOXM	01/600 (A)	13/132 (C/E)
bioMérieux VIDAS Toxo IgG II	Lab code 1.1	0.20	0.36	1.82	0.39	1.98	1.09	0.46	2.37	1.30
	Lab code 2.2	0.36	0.58	1.64	0.63	1.77	1.08	0.98	2.75	1.67
	Lab code 5.3	0.20	0.41	2.01	0.49	2.38	1.18	0.77	3.77	1.87
	Lab code 7.4	0.46	0.67	1.46	0.72	1.57	1.08	0.91	1.98	1.36
	Lab code 8.1	NNV[a]	NNV[a]	1.74	NNV[a]	2.29	1.32	NNV[a]	3.29	1.36
	Lab code 10.2	0.34	0.69	1.83	0.72	1.95	1.06	0.88	2.59	1.41
	Lab code 12.3	0.40	0.67	1.68	0.68	1.70	1.01	0.94	2.34	1.40
	Lab code 13.2	0.10	0.49	5.05	0.57	5.94	1.18	0.73	7.60	1.50
	Lab code 16.1	0.36	0.61	1.72	0.58	1.61	0.94	0.97	2.72	1.58
	GM[b]	0.27	0.55	1.95	0.59	2.14	1.10	0.81	3.01	1.49
	GCV[c]	68.2%	28.5%	44.4%	24.1%	50.5%	10.3%	28.4%	48.5%	12.5%
Abbott ARC Toxo IgG	Lab code 11.2	0.04	0.23	5.84	0.28	7.35	1.26	0.26	6.85	1.17
	Lab code 12.1	NNV[a]	NNV[a]	5.75	NNV[a]	7.14	1.24	NNV[a]	6.68	1.16
	GM[b]			5.80		7.24	1.25		6.77	1.17
Diasorin Liaison Toxo IgG	Lab code 11.5	0.29	0.53	1.83	0.53	1.83	1.00	0.66	2.28	1.25
IgG ELISA	Lab code 4.4	0.84	0.62	0.74	0.55	0.65	0.88	1.54	1.83	2.48
	Lab code 15.1	0.42	0.75	1.79	0.73	1.74	0.97	0.88	2.10	1.18

[a] NNV: TOXM result fell outside assay range and no numerical value was given.
[b] GM: geometric mean.
[c] GCV: geometric coefficients of variation.

Table 6
Results of qualitative assays for TOXM and samples A to F.

Lab code and assay	Antibody detected	Test sample						
		A	B	C	D	E	F	TOXM
4.1 bioMérieux ISAGA	IgM	–	–	+	+	+	+	+
4.5 Immunoblot	IgM	–	–	+	+	+	+	+
5.2 bioMérieux ISAGA	IgM	–	–	+	+	+	+	+
7.1 bioMérieux ISAGA	IgM	–	–	+	+	+	+	+
9.2 ISAGA	IgA	–	–	+	+	+	+	+
9.3 ISAGA	IgM	–	–	+	+	+	+	+
11.1 bioMérieux ISAGA	IgM	–	–	+	+	+	+	+
11.7 LDBio Diagnostics Immunoblot	IgG	+	–	+	+	+	+	+
13.1 bioMérieux VIDAS Toxo Competition	Ig	+	–	+	+	+	+	+

The ratio of the results of coded duplicate samples of 13/132 (C and E) in ELFAs, ELISAs and agglutination assays are shown in Fig. 1. These show that commercial ELFAs and ELISAs have a high level of precision and reproducibility compared to in-house ELISAs. All assay results fell within a two-fold difference relative to 1 (range

0.5–2.0), an indication of good diagnostic precision among participating laboratories.

Based on 10 data sets generated by the bioMérieux VIDAS Toxo IgM ELFA, 13/132 had a GM relative result of 0.75 with a GCV of 4.5% relative to TOXM (Table 4). The relative results for samples D and F for IgM were close to that of 13/132. Two data sets from the Abbott ARC Toxo IgM ELISA and in-house IgM capture ELISAs gave a lower GM values for 13/132 of 0.49 and 0.41 respectively. The relative results for samples D and F for IgM were also close to 13/132 in this assay. The GCVs of the results of samples D and F in the bioMérieux VIDAS Toxo IgM ELFA were lower when 13/132 was used as reference compared to TOXM.

Hansen et al. estimated the unitage of TOXM for IgM to be 3000 units mL^{-1} [4]. In this study, results for IgM assays were reported as indices of signal to noise ratios, with the exception of one IgM capture ELISA by lab 3.2, which reported results in ODs. Based on this data set the potency of IgM for 13/132 relative to TOXM is 0.250, equal to 750 units mL^{-1} or 375 units per ampoule (see Table 4). Due to the limited amount of data and the fact that a unitage for IgM had not been assigned to TOXM, we conclude that 13/132 has a high IgM content and can be considered as a reference reagent but not as an IS for IgM.

The potencies of 13/132 and samples D and F for IgG relative to TOXM, 01/600 and 13/132 are summarised in Table 5. Based on 9 data sets from the bioMérieux VIDAS Toxo IgG II ELFA, 13/132 had a GM potency value of 0.55 with a GCV of 28.5% relative to TOXM and a GM potency value of 1.95 with a GCV of 44.4% relative to 01/600 (Table 5). The relative potency of sample D for IgG was close to 13/132. The relative potencies of sample F for IgG were considerably higher then those for 13/132 in this assay (Table 5). The GCVs of the results of samples D and F in the bioMérieux VIDAS Toxo IgG II ELFA were lower when 13/132 was used as reference compared to TOXM.

Hansen et al. estimated a possible unitage of TOXM for IgG to be 1000 IU mL^{-1} [4]. Based on 12 data sets, we calculated the relative potency of 13/132 for IgG as 0.525 (range 0.23–0.75) this corresponds to 525 units mL^{-1} or 263 units per ampoule (see Table 5). However if the potency of 13/132 for IgG is estimated relative to 01/600 then the GM unitage is considerably lower at 15–116 units mL^{-1} (Table 5). This difference is likely to be caused by the presence of IgM and IgA in TOXM and in 13/132. Both Ig classes will compete with IgG for binding to exposed epitopes in ELISA but not in the dye test, whereas in 01/600 these Ig classes are absent thus allowing a relative high proportion of specific IgG to bind in ELISAs [8]. Due to the fact that a unitage for IgG had not been assigned to TOXM, we conclude that 13/132 can be considered as a reference reagent but not as an IS for IgG.

The avidity of IgG in 13/132 was assessed by ELISA and ELFA and the results of three assays are presented in Table 7. The avidity of IgG in samples C to F was found to be low in 2 out of 3 assays and similar to the avidity of IgG from TOXM. Indeed, Hansen et al. postulated that IgG from TOXM was of low avidity. By contrast the avidity of IgG from 01/600 (A) is considerably higher, pointing to a historic infection. It was noted that low avidity of IgG in combination with a high IgG content contributes to the inter-laboratory variability for 13/132 in diagnostic assays for IgG (see Table 5).

3.3. Qualitative immunoassays

The presence of Ig, IgA, IgG or IgM was also detected by qualitative assays, including capture ELISAs, a competition ELFA, immunoblot assays and ISAGAs. The results are presented in Table 7 and Fig. 2. Results of qualitative assays indicated the presence of anti-Toxoplasma antibodies in TOXM and samples A and C to F and are in agreement with the outcome of quantitative ELISAs and agglutination assays. For example, the immunoblot for IgG (lab code 11.7) confirmed the presence of IgG in TOXM, samples A and C to F by ELISA, HDSA and IFA. The immunoblot for IgM (lab code 4.5) confirmed the presence of IgM in TOXM and samples C to F. Specific IgM bound to a 6 kilo Dalton (kD) antigen of *T. gondii* (see Fig 2). Previous work by Sharma et al. demonstrated that IgM but not IgG from patients with toxoplasmosis reacts with the 6 kD antigen. Hence this reactivity is deemed a diagnostic marker of acute infection [18]. Herbrink et al. showed that the IgM immunoblot can be used to confirm results of IgM capture ELISAs [15]. Data presented here, extend this to IgM detected by IFA and ISAGA.

3.4. Stability studies

Samples of 13/132 were stored for 481 days (~15.3 months) at −20 °C and at elevated temperatures +4 °C, +20 °C, +37 °C and +45 °C. Two samples exposed to each temperature were tested in duplicate in the dye test (lab code 6.1) and in-house IgM ELISA (lab code 6.2 [14]). The potency of the samples, subjected to accelerated thermal degradation, was calculated relative to the samples stored at −20 °C and is given in Table 8.

These results were used to fit Arrhenius equations relating the degradation rate to absolute temperature assuming first-order decay and hence predict the degradation rates when stored at −20 °C [19]. Data from the dye test showed predicted losses of potency of <0.01%, 0.10%, 0.65% and 3.77% per month and 0.05%, 1.16%, 7.27% and 36.0% per year at storage temperatures of −20 °C, +4 °C, +20 °C and +37 °C respectively. Data from the in-house IgM ELISA cannot be directly interpreted as relative potency and are presented for information only.

Table 7
Results of avidity IgG ELISAs for samples A to F and TOXM.

Lab code and assay / Sample tested	11.3 Abbott ARC Toxo IgG avidity (%)[a]	16.1 bioMérieux VIDAS Toxo IgG avidity (OD)[b]	15.2 IgG avidity ELISA (%)[c]
TOXM	22.7 (Low)	0.079 (Low)	27 (Low)
A (01/600)	82.4 (High)	0.496 (High)	59 (High)
C/E (13/132)	34–38.4 (Low)	0.139–0.149 (Low)	42 (Borderline)
D	30.1 (Low)	0.119 (Low)	35 (Borderline)
F	51.9 (Grey zone)	0.218 (Intermediate)	47 (High)

[a] Avidity index: Low <50.0%; Grey zone 50.0–59.9%; High ≥60.0%.
[b] Avidity index: Low <0.200; Intermediate ≥0.200 & <0.300; High ≥0.300.
[c] Avidity index: Low <30; Borderline 30–40; High >40.

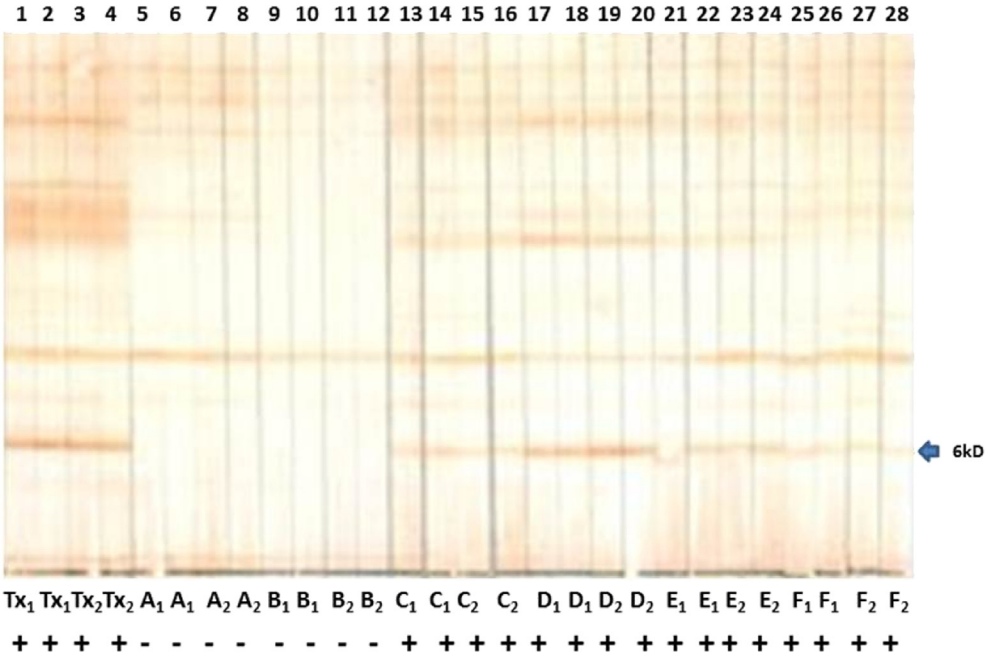

Fig. 2. IgM immunoblot (lab code 4.5) of duplicates of TOXM (Tx) and coded samples A to F, with *T. gondii* strain RH as antigen. The presence of the 6 kD band denotes an IgM positive sample (8). The numbers in subscript indicate the duplicates per sample pack.

Table 8
Test results for samples of candidate IS 13/132 stored at elevated temperatures for approximately 15.3 months.

Lab code and test	Storage temperature	Result[a]			Range	Geometric mean titre	Geometric mean titre relative to −20 °C
		Day 1	Day 2	Day 3			
6.1	−20 °C	500	500	500	500	500	–
Dye test (IU mL^{-1})	+4 °C	500	500	500	500	500	1.00
	+20 °C	500	375	500	250–500	445	0.89
	+37 °C	375	250	250	250–500	281	0.56
	+45 °C	125	125	188	125–250	140	0.28
6.2	−20 °C	91	98	94	91–101	94	–
IgM capture	+4 °C	96	94	96	92–101	95	1.01
ELISA (EU mL^{-1})	+20 °C	85	85	88	81–90	86	0.92
	+37 °C	51	52	60	50–62	55	0.58
	+45 °C	5[b]	18	22	4–23	12	0.13

[a] Average result of two vials is given.
[b] Reconstituted material stored at +45 °C was highly viscous on day 1 prohibiting accurate pipetting of volumes. The viscosity decreased after a 24 h incubation at +4 °C, allowing accurate pipetting of the sample on day 2 and 3.

3.5. Recommendation by the Expert Committee on Biological Standardization

Relative to TOXM, a unitage for Ig by dye test was assigned to 13/132 of 160 IU per ampoule or 320 IU mL^{-1} (GCV of 41.0%). The avidity of IgG is low and comparable to the avidity of IgG from TOXM. 13/132 contains high levels of anti-Toxoplasma IgG and IgM thus allowing calibration in terms of IgG and IgM and its potency falls between TOXM and 01/600. Therefore, 13/132 meets the requirements for a replacement of TOXM as set out by ECBS in 2003 [7]. 13/132 will be a useful addition for the standardisation of Toxoplasma serology and support appropriate clinical management of this disease and 13/132 was proposed as the 4th IS for Antibodies, Human, to *T. gondii* to replace TOXM. A collaborative study report with these findings was submitted to ECBS [20].

ECBS discussed the long term stability of IgM, the current limited use of the dye test compared to former times, and the problems a high IgG titre might cause for current assays. ECBS concluded that the stability issue of IgM is only apparent at accelerated conditions at late time points. Since 13/132 is a replacement standard, comparison by the dye method is justified. ECBS suggested that the unitage proposed for IgG and IgM might be used for information only. However, IgM assays are known to be more challenging to harmonise than IgG assays and a unitage for IgM may be misleading. Thus, for IgM only a high titre should be indicated.

In October 2015, ECBS endorsed the establishment of 13/132 as the 4th IS for Antibodies, Human, to *T. gondii* to replace TOXM, with an assigned unitage of 160 IU per ampoule and an IgG content of 263 U per ampoule relative to TOXM. A high content of IgM was noted. The committee recommended that a potential replacement standard be developed with unitage for IgG and IgM suitable for use with current analytical methods.

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