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1 **Assessment of twelve echovirus virus-neutralisation assays in Europe: recommendations for**
2 **harmonisation of non-polio enterovirus sero-surveillance studies**

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36 **Keywords:** Virus neutralisation assay; enterovirus; echovirus; surveillance; standardisation.
37

38 **Abbreviations:** A549, alveolar carcinoma epithelial cell line; AFM, acute flaccid myelitis; Caco-2,
39 colorectal adenocarcinoma cell line; CPE, cytopathogenic effect; CVA, coxsackievirus A; CVB, coxsackievirus
40 B; DMSO, dimethylsulfoxide; E, echovirus; ELISA, enzyme-linked immunosorbent assay; EMEM, eagle's
41 minimum essential medium; ENPEN, European non-polio enterovirus network; EV, enterovirus; GCV,
42 geometric coefficient of variation; HT29, human B cell lymphoma cell line; IVIG, intravenous
43 immunoglobulins; LLC-MK, rhesus monkey kidney epithelial cell line 2; nAb, neutralising antibodies;

44 NPEV, non-polio enterovirus; RD, rhabdomyosarcoma; SOP, standard operating procedure; TCID50, 50%
45 tissue culture infectious dose; VNA, virus neutralisation assay.

46 **Abstract**

47 Non-polio enteroviruses (NPEV) cause significant disease worldwide. Population-based sero-surveillance,
48 by measuring anti- bodies against specific NPEV types, provides additional information on past
49 circulation and the prediction for future upsurges. Virus neutralisation assays (VNA), the current method
50 of choice for measuring NPEV type specific antibodies, are not entirely standardised. Via the European
51 Non-Polio Enterovirus Network, we organised a VNA quality assessment in which twelve labo- ratories
52 participated. We provided five echovirus (E) types (E1, E18, E30 G2, E30 G6 and E6) and intravenous
53 immunoglobulins (IVIG) as a sample for the NPEV VNA quality assessment. Differences in VNA protocols
54 and neutralising Ab (nAb) titres were found between the participating laboratories with geometric
55 coefficients of variation ranging from 10.3–62.9%. Mixed-effects regression analysis indicated a small
56 but significant effect of type of cell line used. Harmonisation of cell line passage number, however, did
57 not improve variation between laboratories. Calibration by making use of a reference sample, reduced
58 variation between laboratories but differences in nAb titres remained higher than two log₂ dilution steps.
59 In conclusion, sero-surveillance data from different laboratories should be compared with caution and
60 standardised protocols are needed.

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INTRODUCTION

Non-polio enteroviruses (NPEV) are a significant source of disease worldwide [1, 2]. More than 110 NPEV types exist, including coxsackieviruses A and B (CVA and CVB), echoviruses (E) and the numbered enteroviruses (EV). They are the leading cause of viral meningitis in children [3, 4]. Types like EV-D68, EV-A71, E11 and E30 have caused important upsurges of acute flaccid myelitis (AFM), meningoencephalitis and myocarditis respectively [5–9]. The European Non-Polio Enterovirus Network (ENPEN) brings together different stakeholders across Europe to improve surveillance and research of NPEV [10, 11]. In general, molecular surveillance is performed by genotyping EV positive samples obtained from symptomatic cases. As the circulation of NPEV is mainly driven by population immunity, seroprevalence data can help to predict upsurges and the susceptibility of a population to a new viral strain [12]. As ELISA methods suffer from problems like cross-reactivity that do not necessarily reflect immunity, virus neutralisation assays (VNA) are the method of choice to define population immunity. Sero-surveillance studies using this method have been published by different research groups [13–18]. However, measuring type-specific antibodies to the more than 110 NPEV types is complex and cross-neutralisation has been reported [19]. Furthermore, VNA procedures are not entirely standardised, which could influence assay neutralising Ab (nAb) titres and hamper comparison of data. With this study, we assess capacity for standardised sero-surveillance studies within the ENPEN network.

87

88 **METHODS**

89 *Survey VNA*

90 An invitation to participate in the study was sent out to different ENPEN laboratories in April 2021.
91 Twelve laboratories from Bulgaria, the Czech Republic, Finland, France (n=2), Germany, Croatia, the
92 Netherlands, Portugal, Spain, Romania, and the United Kingdom (England) agreed to participate in
93 the first quality assessment reported here. Participating laboratories were asked to provide their VNA
94 standard operating procedure (SOP) (summarised in Table 1).

95

96 *First quality assessment*

97 Five echovirus types E1 (Virus [V]1), E18 (V2), E30 G2 (genogroup II) (V3), E30 G6 (genogroup III) (V4)
98 and E6 (V5) (Table S1, available in the online version of this article) and four individual aliquots of IVIG
99 (Nanogam, lot number 17D29H462A, 2017, Prothya Biosolutions, the Netherlands) dilutions 1 : 1
100 (Serum [S]1), 1 : 4 (S2), 1 : 16 (S3), 1 : 64 (S4) were sent at room temperature to the participating
101 laboratories using the V and S codes. In case the estimated transport time took >48 h (laboratories 3,
102 4 and 12), transportation was done on dry ice. An instruction form was provided including the
103 guideline to store virus stocks at -80°C and samples at -20°C until performance of the assay.
104 Participating laboratories were asked to determine the 50% tissue culture infectious dose (TCID₅₀)
105 ml⁻¹ of V1–V5 and nAb titres according to the laboratories' own VNA SOP against V1–V5 in S1 and
106 against V5 in S2–4. The nAb results were collected on a standard form (added as File S1). Inter-
107 laboratory variation of nAb titres was analysed by calculating geometric coefficients of variation (CV)

108 of the log₂ transformed nAb titres against V1–V5 in S1. To assess intra-laboratory variation and to
109 evaluate the possibility to use IVIG as a reference sample the linearity of the provided nAb titres
110 against V5 in the IVIG dilution series was analysed for each laboratory by calculating Spearman's rank
111 correlation coefficients.

113 *Second quality assessment: harmonisation and calibration*

114 The second quality assessment aimed to reduce inter-laboratory variability by introducing
115 reference samples, to be used as calibrators, and by standardising the cell line (RD99) and day of
116 read-out. Ten of the twelve initial laboratories from Bulgaria, Finland, France (n=2), Germany, the
117 Netherlands, Portugal, Spain, Romania, and the United Kingdom (England) participated in the second
118 assessment. Two echovirus types (E30 G2 [V3] and E30 G6 [V4]) and three IVIG dilutions 1 : 1 (S1), 1 :
119 4 (S2), 1 : 16 (S3) were sent to the participating laboratories. A reference serum (R) dilution series 1 : 8
120 (R1), 1 : 16 (R2), 1 : 32 (R3), and 1 : 64 (R4) and a RD cell line (RD99 cell line passage 235) frozen in
121 dimethylsulfoxide (DMSO) 10% and foetal bovine serum 10% was also provided. All materials were
122 sent on dry ice. Three of the participating laboratories (2, 10 and 11) experienced problems with
123 culturing or contamination of the frozen cell line stock. For these laboratories a flask with a
124 monolayer in Eagle's Minimum Essential Medium (EMEM) and FBS 8% was sent at room
125 temperature. An instruction form was provided including the guideline to store virus stocks and
126 cell lines at -80°C and samples at -20°C until performance of the assay. We asked to determine nAb
127 titres in S1–S3 against V3 and V4 according to the laboratories' VNA SOP with the provided RD99 and
128 laboratories' proper cell lines and to perform an additional CPE read-out at day 7. We asked to
129 determine nAb titres in R1–R4 according to the laboratories' VNA SOP with the provided RD99 cell

130 line, and to perform an additional CPE read-out at day 7. As we did not know the real nAb titre of
131 the reference sample R, we took the median of measured nAb titres in R1 of all participating
132 laboratories and we calculated nAb titres in R2–R4 by considering their dilution factors. For each
133 laboratory, the measured log₂ transformed nAb titres in S1–S3 were calibrated according to the
134 regression formula calculated based on R1–R4 (Fig. S1). By calculating the geometric CV values, the
135 dispersion was compared between measured (non-calibrated) and calibrated nAb titres in S1–S3.

136 137 *Mixed-effects regression analysis*

138 To evaluate the individual effects of different VNA variables on the nAb titres, we performed a mixed-
139 effects regression analysis. Log₂-transformed nAb titres were predicted by cell line, initial TCID₅₀, virus
140 inactivation, virus/sample co-incubation (hours) and day of read-out as fixed effect independent
141 variables. To avoid overfitting of the model different cell line passages were not considered in the
142 model. Cell lines were divided into two categories: standardised cell line (RD99) or other cell lines.
143 Virus and laboratory were added as random effects. The significance of each independent fixed-effect
144 variable was calculated by comparing the likelihood of the model with and without the respective
145 variable via an ANOVA likelihood ratio test.

146 147 *Figures and statistics*

148 All figures and statistics were conducted in R version 4.0.3 in R studio version 2023.03.1+446 with
149 packages ggplot2, dplyr, PKNCA and lme4 [20].

151 RESULTS

152 *VNA characteristics*

153 Similarities and differences in VNA characteristics are presented in Table 1. All laboratories
154 except laboratory seven co-incubated 100 TCID₅₀ of the virus with sample dilutions, incubation
155 lengths ranging from 1 to 3 h. More than half of the participants performed serum sample
156 inactivation (7/12). There were differences in type of cell lines used, whether they were used in
157 suspension or monolayer and number of cells added. Nine of the twelve participating laboratories
158 used RD as a cell line for all included echovirus types. Seven of the twelve participating laboratories
159 used a monolayer of cells. The number of cells, when counted, ranged from 14400 to 25000 cells per
160 well. Assay incubation length ranged from 3 to 7 days. All participating laboratories used CPE or
161 Crystal Violet (as an indication of CPE absence) as a read-out. Two main methods of nAb titre
162 calculation were used: the last dilution with 100% neutralization (both wells with less than 50% CPE)
163 (n=4) and the Reed Muench method (n=7) [21].

164 165 *First quality assessment*

166 The first quality assessment aimed to evaluate the inter-laboratory variability of VNA titres.
167 Differences of approximately three log₁₀ TCID₅₀ ml⁻¹ were observed when culturing the echovirus
168 types in the laboratories' conditions (Table S2). A wide range of nAb titres was observed between
169 laboratories, especially against E1 and E18 (Fig. 1a and Table S3). Neutralising Ab titres ranged from 4 to
170 >1024 for E1 and E18, from 6 to >1024 for E30 G2, from 8 to 1024 for E30 G6 and from 64 to 1024 for E6.
171 The geometric CV for E1, E18, E30 G2, E30 G6 and E6 were 51, 62.9, 40, 31.7 and 17.2% respectively. Five
172 of the twelve participating laboratories reported back titration results, of which the majority were

173 between $10^{1.5}$ and $10^{2.5}$ TCID₅₀ per well (Table S3). Spearman's rank correlation coefficients of
174 nAb titres in the IVIG dilution series, reported by nine of the twelve participating laboratories, ranged
175 from -0.95 to -1 (Fig. 1b)

177 *Second quality assessment*

178 Our second quality assessment aimed to reduce inter-laboratory variability by introducing reference
179 samples to be used as calibrators and by standardizing the cell line (RD99) and day of read-out.

180 Neutralising Ab titres against E30 G2 ranged from 256 to 1024 and 256 to 2048 with the respective
181 laboratories' proper and RD99 cell lines, corresponding to a geometric CV of 8.2 and 10.5% respectively
182 (Fig. S2). Neutralising Ab titres against E30 G6 ranged from 128 to 640 and 128 to 1024 with the
183 respective laboratories' proper and RD99 cell lines, corresponding to a geometric CV of 13.1 and
184 13.9%, respectively. Although a large spread of initial TCID₅₀ results between different laboratories
185 was observed, there were no major intra-laboratory differences in initial TCID₅₀ results between the
186 provided RD99 and the laboratories' cell lines (mostly RD cell lines) (Table S3). An exception was
187 laboratory nine, where the TCID₅₀ ml⁻¹ of E30 G2 was tenfold higher with the Caco-2 cell line than
188 with the RD99 cell line.

189 Back titration results ranged from 4 to 3162 TCID₅₀ per well (Table S4). A very weak correlation was
190 found between nAb titres and TCID₅₀ back titration results ($r=-0.18$) (Fig. S3). There were no intra-
191 laboratory differences in nAb titres between different days of read-out, ranging from 3 to 7 days.
192 Spearman's rank correlation coefficients in R1–R4 ranged from 0.8 to 1. Calibration, according to the
193 regression formula calculated based on R1–R4, reduced the dispersion of nAb titres in S1–S3 with in
194 general lower geometric CVs between laboratories for calibrated nAb titres compared to measured

195 nAb titres. Several outliers remained (Fig. 2 and Table 2).

196
197 *Mixed-effects regression analysis*

198 Since no clear cause of the nAb titre variation could be identified after the first and second quality
199 assessment, we analysed the independent effects of different VNA variables (cell line, initial TCID₅₀,
200 virus inactivation, virus/sample co-incubation [hours]
201 and day of read-out), controlling for the random effect of virus (E1, E18, E30 G2, E30 G6 or E6) and
202 participating laboratory. Cell line (RD99 or other) independently affected nAb titre (P=0.02651),
203 lowering the nAb titre by 0.804190 log₂ or 1.75 when using RD99. Although statistically significant,
204 this is less than what is considered normal variation (one log₂ dilution step). No other significant
205 effects were observed.

206
207 **DISCUSSION**

208 In this study, we tried to investigate the comparability of sero-surveillance data by organizing a
209 VNA quality assessment and comparing different SOPs of twelve laboratories of the ENPEN network
210 across Europe. A wide variation of nAb titres was found, with differences ranging from 16- to 256-
211 fold. For two of the five viruses tested (E1 and E18), nAb titres ranged from negative (below cut-off)
212 to >1024 (above cut-off). Along with differences in nAb titres, there were differences in VNA
213 characteristics like sample inactivation, incubation time, use of cell line (type, suspension/monolayer
214 and number of cells), day of read-out and nAb titre calculation. This means that results from sero-
215 surveillance studies between different laboratories cannot be compared for these echoviruses, and

216 most probably for other NPEV neither. Inter-laboratory differences between VNA nAb titres can be
217 improved using a reference or an international standard serum sample [22]. In our study, inter-
218 laboratory variation improved after using a type-specific animal serum sample as a calibrator but
219 remained considerable. It is possible that non-systematic errors, such as calculation or pipetting
220 errors and differences in read-out interpretation caused some of the outliers. VNA are laborious
221 assays susceptible to inter-individual variation. Further automatisation and standardisation for
222 example of read-out techniques (measurement of viral antigens or cell death using ELISA) could
223 potentially reduce inter-assay variation [23]. With more than 110 enterovirus types, providing a
224 reference sample for every individual type would be a major challenge. Therefore, we evaluated if
225 IVIG could serve as a universal NPEV reference sample. We found good linearity against all analysed
226 echovirus types, making IVIG a possible and more practical alternative to type-specific reference
227 serum samples. Importantly, IVIG of one identical batch should be used. Associations between nAb
228 titres and different VNA variables were explored. High differences in echovirus TCID₅₀ results
229 between different laboratories were found, and preliminary analysis suggested a possible association
230 between nAb titres and cell line passage, day of read- out and back titration results. We therefore
231 standardised the cell line and day of read-out. Mixed-effects regression analysis indicated a small but
232 significant effect of type of cell line used. Our hypothesis was that differences in TCID₅₀ results for the
233 same quantity of virus are caused by differences in cell line susceptibility. This results in differences of
234 virus added to the VNA and thus resulting in differences in nAb titres [24]. In the second assessment
235 however, TCID₅₀ results did not differ between the laboratories' RD cell lines and the provided RD99
236 cell line. Moreover, the dispersion of nAb titres was not reduced by harmonising the cell line. We did
237 see a substantial difference between the TCID₅₀ results on Caco-2 and RD99. Accordingly, the cell line
238 type probably plays a more important role than the number of cell line passage. As most laboratories

239 used RD cell lines it could be that we underestimated the effect of harmonising the cell line.
240 Furthermore, other unknown factors in the treatment of these cell lines could play a role. All
241 possible factors causing variation need to be addressed. While awaiting standardisation via
242 international agreement on assay procedures and reference samples (like for poliovirus and EV-A71)
243 [25, 26] laboratories could, when performing sero-surveillance studies, send a subset of the samples
244 and the respective virus strain to another laboratory for confirmation. Or if several laboratories are
245 involved in a sero-surveillance study, the same EV strain and a reference sample should be agreed
246 upon.

247 There are several limitations to our study. One is that only a limited number of echovirus types were
248 analysed. Secondly, there were too many missing back titration results to include this variable in the
249 mixed-effects regression analysis. Furthermore, it is possible that other unknown VNA variables,
250 including variations in transport conditions, were not included in the analysis. Finally, as no
251 international standards exist for the included echoviruses, we had no golden standard to compare nAb
252 titres with. Therefore, the median nAb titre of all participating laboratories in the reference sample
253 was considered for normalization. In conclusion, we conducted an echovirus VNA quality assessment
254 in different European countries. Although providing a reference serum sample can improve
255 standardisation, comparison between sero-surveillance studies performed by different laboratories
256 should be done with caution and additional research is necessary to understand the effect of different
257 VNA variables. Based on this knowledge practical recommendation to perform VNA should be further
258 explored.

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265

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274

275 **Author contributions**

276 K.C., K.B., K.W., J.L.M., G.K. and H.v.E.: Study design; G.K., H.v.E., E.K., I.S., M.J., L.N.G., I.G., A.S., S.D., S.B., M.C., I.T., Z.H.,
277 P.S. and E.H.: Experiments; K.C. and M.K.: Data analysis; K.C., K.B., K.W., J.L.M., D.P., H.H., J.L.B. and M.B.: Conclusions
278 and writing.

279

280 **Conflicts of interest**

281 The authors declare that the research was conducted in the absence of any commercial or financial
282 relationships that could be construed as a potential conflict of interest.

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Table 1. VNA characteristics of different participating laboratories. Origin of cell lines:

RD=Rhabdomyosarcoma, HT29=Human B cell lymphoma cell line, A549=Alveolar carcinoma epithelial cell line, Caco-2=Colorectal adenocarcinoma cell line, LLC- MK=Rhesus Monkey Kidney Epithelial Cell line 2. Abbreviations: TCID₅₀=50% tissue culture infectious dose, CPE=Cytopathogenic effect, nAb=Neutralising antibody

Participating laboratory	Cell line	Mono-layer (M)/ Suspension(S)	No. of cells/well	TCID ₅₀ virus/well added	Serum sample inactivation	Virus /sample co-incubation before adding cells (hours)	Assay read-out	Method of nAb titre calculation	Incubation length (days)	Chloroform used
1	HT29, RD, A549	S	Not counted	100	Yes	1	CPE	Reed Muench	7	Yes (E18)
2	RD	M	18000	100	No	3	CPE	Reed Muench	6	No
3	RD	M	15000	100	No	2	CPE	Last dilution with 100% neutralisation	3	No
4	RD	M	20000	100	No	1	CPE	Improved Karber Method	5	No
5	RD	S	15000	100	Yes	1	CPE	Reed Muench	3	No
6	RD	M	25000	100	No	3	CPE	Reed Muench	7	No
7	RD	M	25000	200	No	3	Crystal violet	Reed Muench	3	No
8	RD	S	15000	100	No	3	Crystal violet	Reed Muench	6	No
9	Caco-2	S	Not counted	100	Yes	3	CPE		5	No
10	RD	S	14400	100	Yes	1.5	CPE	Last dilution with 100% neutralisation	6	No
11	RD	M	Not counted	100	No	3	CPE	Last dilution with 100% neutralisation	4	No
12	RD, LLC-MK2	S	15000 (10000 LLC-MK2)	100	Yes	1	CPE	Last dilution with 100% neutralisation	RD 3, LLC-MK2 4	No

Table 2. Geometric coefficients of variation (GCV) of nAb titres determined by ten laboratories in
 IVIG dilutions 1:1 (S1), 1:4 (S2), 1:16 (S3) before and after calibration

	E30 G2		E30 G6	
	Measured GCV (%)	Calibrate GCV (%)	Measured GCV (%)	Calibrated GCV (%)
S1	10.3	9.7	15.6	15.8
S2	24.5	11.6	50.6	16.6
S3	60	17	37.8	31.5

Fig. 1. Neutralising Ab titres by participating laboratories according to their proper SOP in (a) S1 against echovirus types E1, E18, E30 G2, E30 G6 and E6 (b) dilution series S1–S4 against E6.

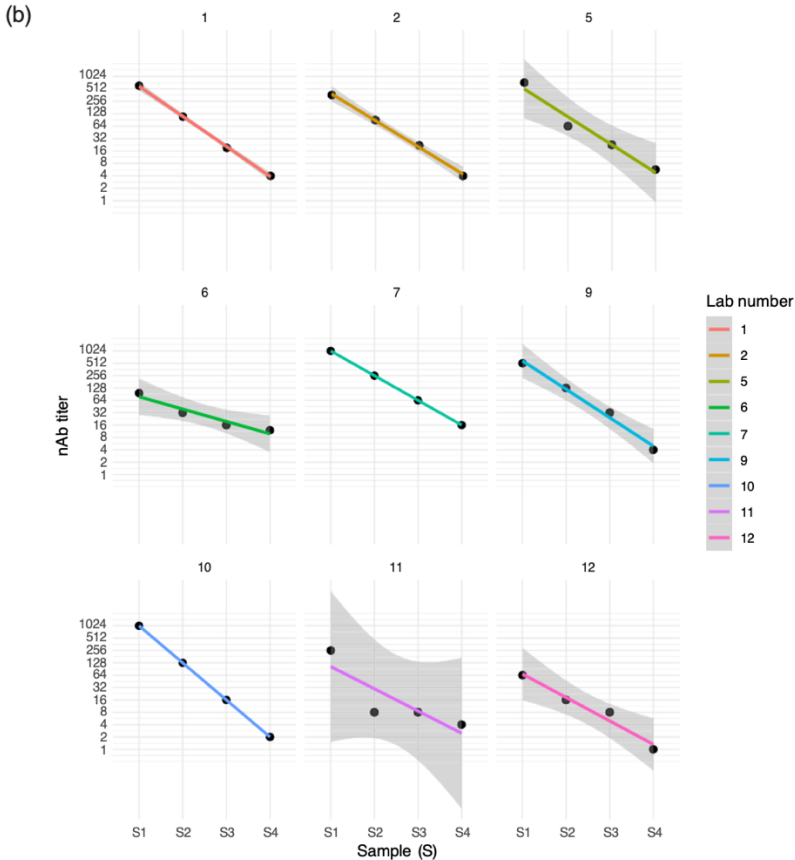
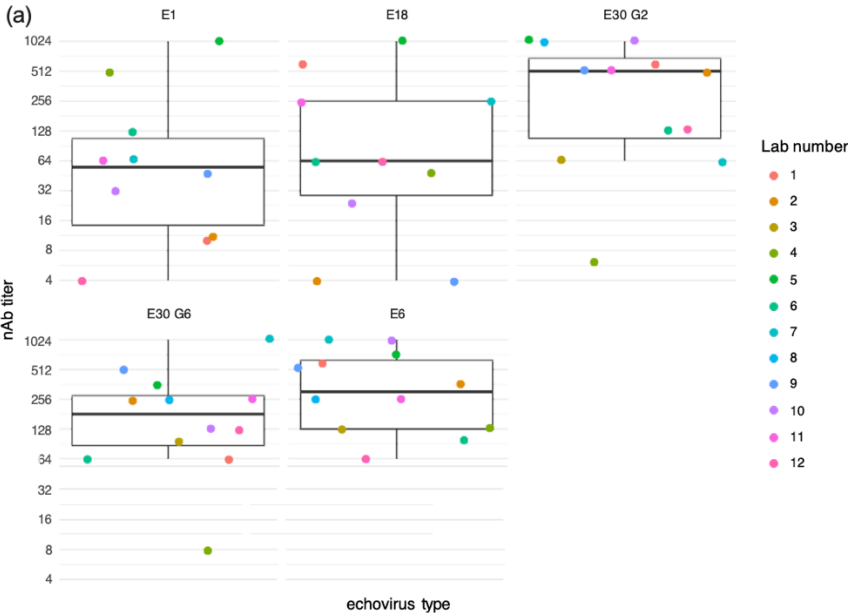


Fig. 2. Comparison between measured and calibrated nAb titres against E30 G2 (a) and E30 G6 (b) with the provided RD-99 cell line passage and reference sample.

