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

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Abstract

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

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Abbreviations: A549, alveolar carcinoma epithelial cell line; AFM, acute flaccid myelitis; Caco-2, colorectal adenocarcinoma cell line; CPE, cytopathogenic effect; CVA, coxsackievirus A; CVB, coxsackievirus B; DMSO, dimethylsulfoxide; E, echovirus; ELISA, enzyme-linked immunosorbent assay; EMEM, eagle's minimum essential medium; ENPEN, European non-polio enterovirus network; EV, enterovirus; GCV, geometric coefficient of variation; HT29, human B cell lymphoma cell line; IVIG, intravenous immunoglobulins; LLC-MK, rhesus monkey kidney epithelial cell line 2; nAb, neutralising antibodies; NPEV, non-polio enterovirus; RD, rhabdomyosarcoma; SOP, standard operating procedure; TCID50, 50% tissue culture infectious dose; VNA, virus neutralisation assay.

The raw data supporting the conclusions of this article will be made available by the authors upon request.

Three supplementary figures and five supplementary tables are available with the online version of this article.

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.

METHODS

Survey VNA

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

First quality assessment

Five echovirus types E1 (Virus [V]1), E18 (V2), E30 G2 (genogroup II) (V3), E30 G6 (genogroup III) (V4) and E6 (V5) (Table S1, available in the online version of this article) and four individual aliquots of IVIG (Nanogam, lot number 17D29H462A, 2017, Prothya Biosolutions, the Netherlands) dilutions 1:1 (Serum [S]1), 1:4 (S2), 1:16 (S3), 1:64 (S4) were sent at room temperature to the participating laboratories using the V and S codes. In case the estimated transport time took >48 h (laboratories 3, 4 and 12), transportation was done on dry ice. An instruction form was provided including the guideline to store virus stocks at −80°C and samples at −20°C until performance of the assay. Participating laboratories were asked to determine the 50% tissue culture infectious dose ($TCID_{50}$) ml^{-1} of V1–V5 and nAb titres according to the laboratories' own VNA SOP against V1–V5 in S1 and against V5 in S2–4. The nAb results were collected on a standard form (added as File S1). Inter-laboratory variation of nAb titres was analysed by calculating geometric coefficients of variation (CV) of the log2 transformed nAb titres against V1–V5 in S1. To assess intra-laboratory variation and to evaluate the possibility to use IVIG as a reference sample the linearity of the provided nAb titres against V5 in the IVIG dilution series was analysed for each laboratory by calculating Spearman's rank correlation coefficients.

Second quality assessment: harmonisation and calibration

The second quality assessment aimed to reduce inter-laboratory variability by introducing reference samples, to be used as calibrators, and by standardising the cell line (RD99) and day of read-out. Ten of the twelve initial laboratories from Bulgaria, Finland, France (*n*=2), Germany, the Netherlands, Portugal, Spain, Romania, and the United Kingdom (England) participated in the second assessment. Two echovirus types (E30 G2 [V3] and E30 G6 [V4]) and three IVIG dilutions 1:1 (S1), 1:4 (S2), 1:16 (S3) were sent to the participating laboratories. A reference serum (R) dilution series 1:8 (R1), 1:16 (R2), 1:32 (R3), and 1:64 (R4) and a RD cell line (RD99 cell line passage 235) frozen in dimethylsulfoxide (DMSO) 10% and foetal bovine serum 10% was also provided. All materials were sent on dry ice. Three of the participating laboratories (2, 10 and 11) experienced problems with culturing or contamination of the frozen cell line stock. For these laboratories a flask with a monolayer in Eagle's Minimum Essential Medium (EMEM) and FBS 8% was sent at room temperature. An instruction form was provided including the guideline to store virus stocks and cell lines at −80°C and samples at −20°C until performance of the assay. We asked to determine nAb titres in S1–S3 against V3 and V4 according to the laboratories' VNA SOP with the provided RD99 and laboratories' proper cell lines and to perform an additional CPE read-out at day 7. We asked to determine nAb titres in R1–R4 according to the laboratories' VNA SOP with the provided RD99 cell line, and to perform an additional CPE read-out at day 7. As we did not know the real nAb titre of the reference sample R, we took the median of measured nAb titres in R1 of all participating laboratories and we calculated nAb titres in R2–R4 by considering their dilution factors. For each laboratory, the measured log2 transformed nAb titres in S1–S3 were calibrated according to the regression formula calculated based on R1–R4 (Fig. S1). By calculating the geometric CV values, the dispersion was compared between measured (non-calibrated) and calibrated nAb titres in S1–S3.

Table 1. VNA characteristics of different participating laboratories

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

*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: $TCID_{50}$=50% tissue culture infectious dose, CPE=Cytopathogenic effect, nAb=Neutralising antibody

Mixed-effects regression analysis

To evaluate the individual effects of different VNA variables on the nAb titres, we performed a mixed-effects regression analysis. Log2-transformed nAb titres were predicted by cell line, initial $TCID_{50}$, virus inactivation, virus/sample co-incubation (hours) and day of read-out as fixed effect independent variables. To avoid overfitting of the model different cell line passages were not considered in the model. Cell lines were divided into two categories: standardised cell line (RD99) or other cell lines. Virus and laboratory were added as random effects. The significance of each independent fixed-effect variable was calculated by comparing the likelihood of the model with and without the respective variable via an ANOVA likelihood ratio test.

Figures and statistics

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

RESULTS

VNA characteristics

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

First quality assessment

The first quality assessment aimed to evaluate the inter-laboratory variability of VNA titres. Differences of approximately three log10 $TCID_{50}$ ml^{-1} were observed when culturing the echovirus types in the laboratories' conditions (Table S2). A wide range of nAb titres was observed between laboratories, especially against E1 and E18 (Fig. 1a and Table S3). Neutralising Ab titres ranged from 4 to >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. The geometric CV for E1, E18, E30 G2, E30 G6 and E6 were 51, 62.9, 40, 31.7 and 17.2% respectively. Five of the twelve participating laboratories reported back titration results, of which the majority were between $10^{\wedge 1.5}$ and $10^{\wedge 2.5}$ $TCID_{50}$ per well (Table S3). Spearman's rank correlation coefficients of nAb titres in the IVIG dilution series, reported by nine of the twelve participating laboratories, ranged from −0.95 to −1 (Fig. 1b)

Second quality assessment

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

Neutralising Ab titres against E30 G2 ranged from 256 to 1024 and 256 to 2048 with the respective laboratories' proper and RD99 cell lines, corresponding to a geometric CV of 8.2 and 10.5% respectively (Fig. S2). Neutralising Ab titres against E30 G6 ranged from 128 to 640 and 128 to 1024 with the respective laboratories' proper and RD99 cell lines, corresponding to a geometric CV of 13.1 and 13.9%, respectively. Although a large spread of initial $TCID_{50}$ results between different laboratories was observed, there were no major intra-laboratory differences in initial $TCID_{50}$ results between the provided RD99 and the laboratories' cell lines (mostly RD cell lines) (Table S3). An exception was laboratory nine, where the $TCID_{50}$ ml^{-1} of E30 G2 was tenfold higher with the Caco-2 cell line than with the RD99 cell line.

Back titration results ranged from 4 to 3162 $TCID_{50}$ per well (Table S4). A very weak correlation was found between nAb titres and $TCID_{50}$ back titration results (r=−0.18) (ig. S3). There were no intra-laboratory differences in nAb titres between different days of read-out, ranging from 3 to 7 days. Spearman's rank correlation coefficients in R1–R4 ranged from 0.8 to 1. Calibration, according to the regression formula calculated based on R1–R4, reduced the dispersion of nAb titres in S1–S3 with in general lower geometric CVs between laboratories for calibrated nAb titres compared to measured nAb titres. Several outliers remained (Fig. 2 and Table 2).

Mixed-effects regression analysis

Since no clear cause of the nAb titre variation could be identified after the first and second quality assessment, we analysed the independent effects of different VNA variables (cell line, initial $TCID_{50}$, virus inactivation, virus/sample co-incubation [hours]

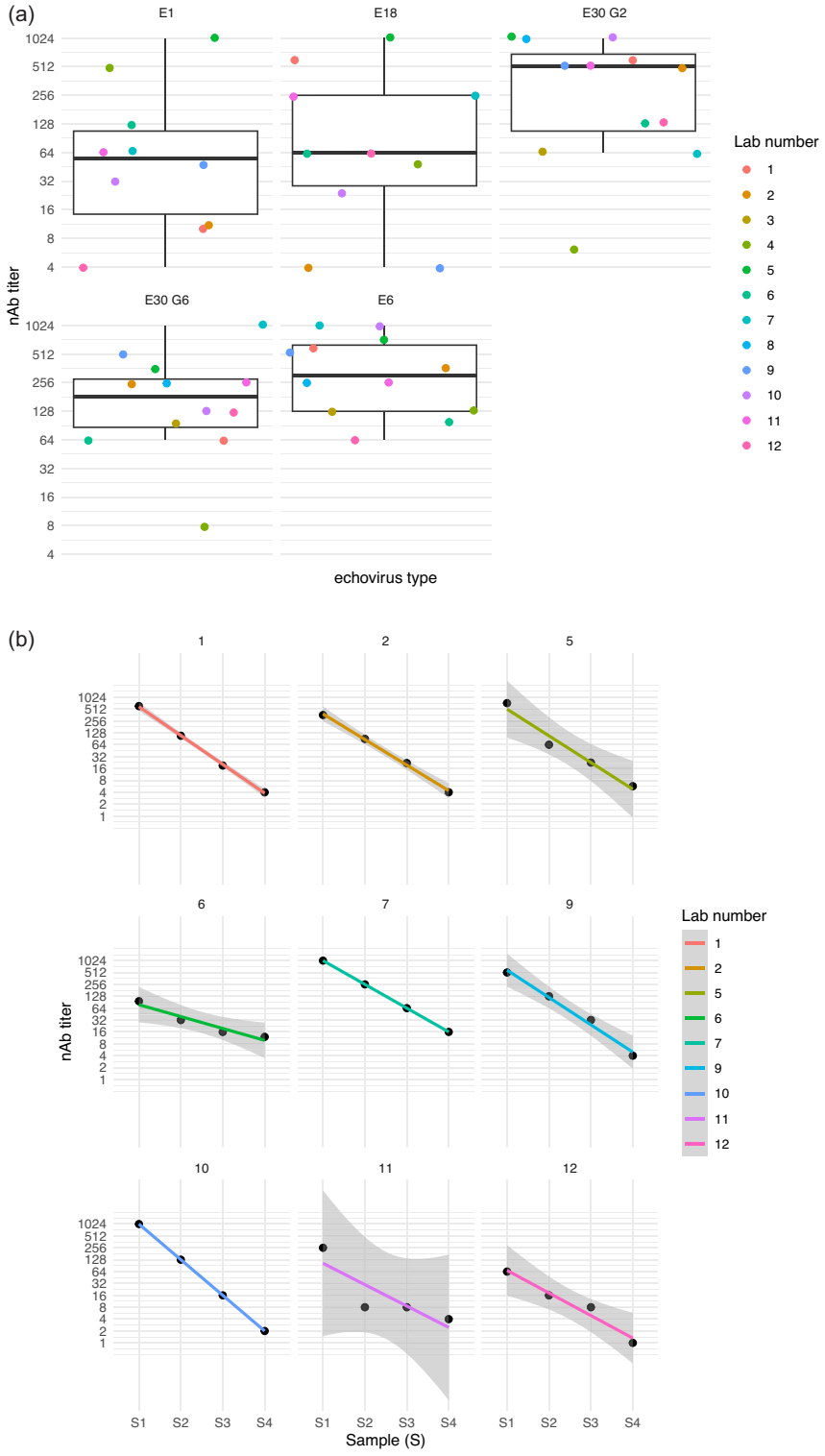

Fig. 1. Neutralising Ab titres by participating laboratories according to their proper SOP in (a) S1 against echovirus types E1, E18, E30 G2, E30 G6 and E6 (b) dilution series S1–S4 against E6.

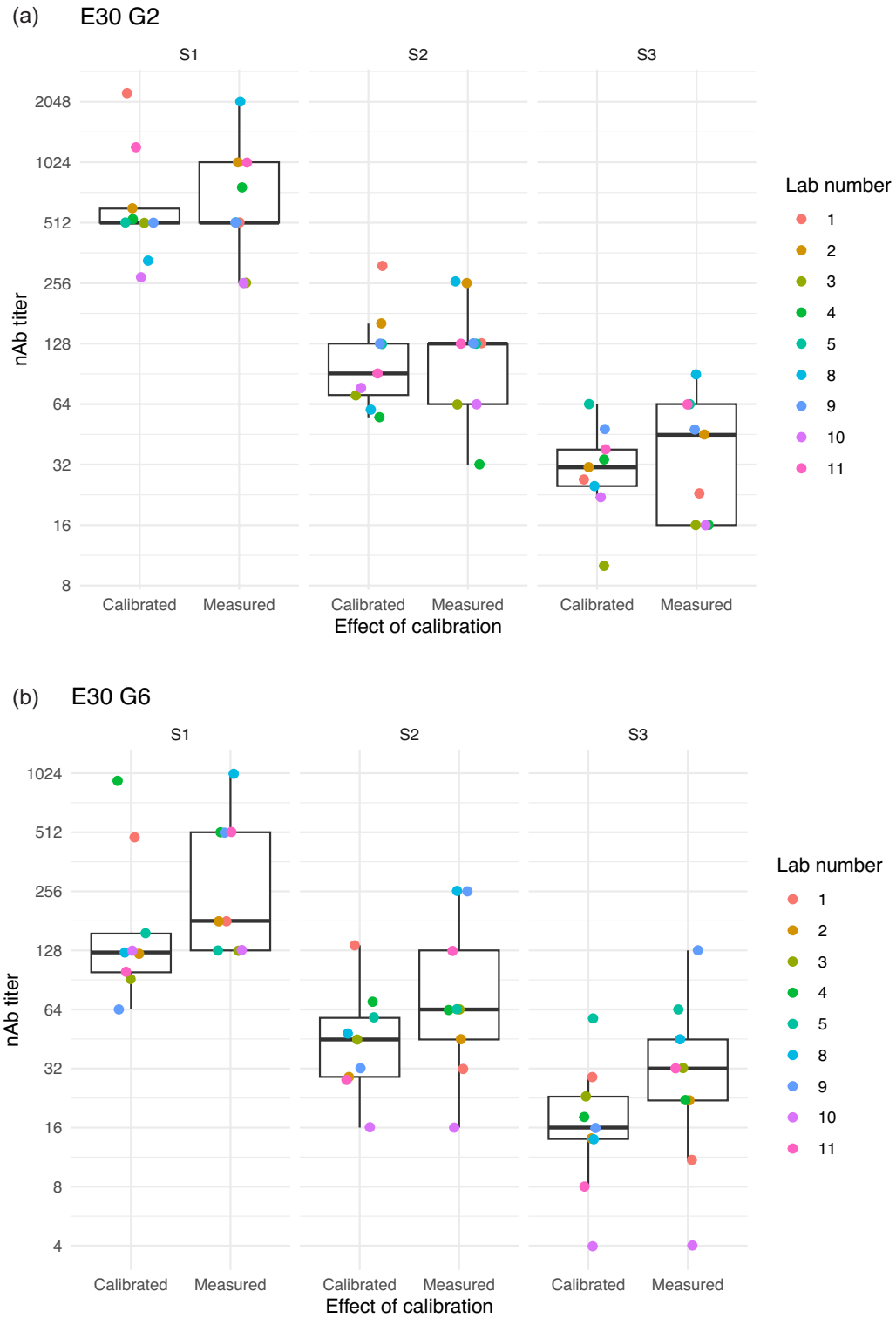

Fig. 2. Comparison between measured and calibrated nAb titres against E30 G2 (a) and E30 G6 (b) with the provided RD-99 cell line passage and reference sample.

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

and day of read-out), controlling for the random effect of virus (E1, E18, E30 G2, E30 G6 or E6) and participating laboratory. Cell line (RD99 or other) independently affected nAb titre (P=0.02651), lowering the nAb titre by 0.804190 log2 or 1.75 when using RD99. Although statistically significant, this is less than what is considered normal variation (one log2 dilution step). No other significant effects were observed.

DISCUSSION

In this study, we tried to investigate the comparability of sero-surveillance data by organizing a VNA quality assessment and comparing different SOPs of twelve laboratories of the ENPEN network across Europe. A wide variation of nAb titres was found, with differences ranging from 16- to 256-fold. For two of the five viruses tested (E1 and E18), nAb titres ranged from negative (below cut-off) to >1024 (above cut-off). Along with differences in nAb titres, there were differences in VNA characteristics like sample inactivation, incubation time, use of cell line (type, suspension/monolayer and number of cells), day of read-out and nAb titre calculation. This means that results from sero-surveillance studies between different laboratories cannot be compared for these echoviruses, and most probably for other NPEV neither. Inter-laboratory differences between VNA nAb titres can be improved using a reference or an international standard serum sample [22]. In our study, inter-laboratory variation improved after using a type-specific animal serum sample as a calibrator but remained considerable. It is possible that non-systematic errors, such as calculation or pipetting errors and differences in read-out interpretation caused some of the outliers. VNA are laborious assays susceptible to inter-individual variation. Further automatisation and standardisation for example of read-out techniques (measurement of viral antigens or cell death using ELISA) could potentially reduce inter-assay variation [23]. With more than 110 enterovirus types, providing a reference sample for every individual type would be a major challenge. Therefore, we evaluated if IVIG could serve as a universal NPEV reference sample. We found good linearity against all analysed echovirus types, making IVIG a possible and more practical alternative to type-specific reference serum samples. Importantly, IVIG of one identical batch should be used. Associations between nAb titres and different VNA variables were explored. High differences in echovirus $TCID_{50}$ results between different laboratories were found, and preliminary analysis suggested a possible association between nAb titres and cell line passage, day of read-out and back titration results. We therefore standardised the cell line and day of read-out. Mixed-effects regression analysis indicated a small but significant effect of type of cell line used. Our hypothesis was that differences in $TCID_{50}$ results for the same quantity of virus are caused by differences in cell line susceptibility. This results in differences of virus added to the VNA and thus resulting in differences in nAb titres [24]. In the second assessment however, $TCID_{50}$ results did not differ between the laboratories' RD cell lines and the provided RD99 cell line. Moreover, the dispersion of nAb titres was not reduced by harmonising the cell line. We did see a substantial difference between the $TCID_{50}$ results on Caco-2 and RD99. Accordingly, the cell line type probably plays a more important role than the number of cell line passage. As most laboratories used RD cell lines it could be that we underestimated the effect of harmonising the cell line. Furthermore, other unknown factors in the treatment of these cell lines could play a role. All possible factors causing variation need to be addressed. While awaiting standardisation via international agreement on assay procedures and references samples (like for poliovirus and EV-A71) [25, 26] laboratories could, when performing sero-surveillance studies, send a subset of the samples and the respective virus strain to another laboratory for confirmation. Or if several laboratories are involved in a sero-surveillance study, the same EV strain and a reference sample should be agreed upon.

There are several limitations to our study. One is that only a limited number of echovirus types were analysed. Secondly, there were too many missing back titration results to include this variable in the mixed-effects regression analysis. Furthermore, it is possible that other unknown VNA variables, including variations in transport conditions, were not included in the analysis. Finally, as no international standards exist for the included echoviruses, we had no golden standard to compare nAb titres with. Therefore, the median nAb titre of all participating laboratories in the reference sample was considered for normalization. In conclusion, we conducted an echovirus VNA quality assessment in different European countries. Although providing a reference serum sample can improve standardisation, comparison between sero-surveillance studies performed by different laboratories

should be done with caution and additional research is necessary to understand the effect of different VNA variables. Based on this knowledge practical recommendation to perform VNA should be further explored.

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Author contributions
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., 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 and writing.

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

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