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Evaluation and validation of a commercial ELISA versus the *in vitro* toxin neutralization assay for determination of diphtheria anti-toxin in human serum

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Abstract

Introduction. Diphtheria is a potentially life-threatening infection and remains endemic in many low- and middle-income countries (LMICs). A reliable, low-cost method for serosurveys in LMICs is warranted to estimate the accurate population immunity to control diphtheria.

Hypothesis/Gap Statement. The correlation between the ELISA results against diphtheria toxoid and the gold standard diphtheria toxin neutralization test (TNT) values is poor when ELISA values are <0.1 IU ml^{-1}, which results in inaccurate estimates of susceptibility in populations when ELISA is used for measuring antibody levels.

Aim. To explore methods to accurately predict population immunity and TNT-derived anti-toxin titres from ELISA anti-toxoid results.

Methodology. A total of 96 paired serum and dried blood spot (DBS) samples collected in Vietnam were used for comparison of TNT and ELISA. The diagnostic accuracy of ELISA measurement with reference to TNT was assessed by area under the receiver operating characteristic (ROC) curve (AUC) and other parameters. Optimal ELISA cut-off values corresponding to TNT cut-off values of 0.01 and 0.1 IU ml^{-1} were identified by ROC analysis. A method based on the multiple imputation approach was also applied to estimate TNT measurements in a dataset that only included ELISA results. These two approaches were then applied to ELISA results previously generated from 510 subjects in a serosurvey in Vietnam.

Results. The ELISA results on DBS samples showed a good diagnostic performance compared to TNT. The cut-off values for ELISA measurement corresponding to the TNT cut-off values of 0.01 IU ml^{-1} were 0.060 IU ml^{-1} in serum samples, and 0.044 IU ml^{-1} in DBS samples. When a cut-off value of 0.06 IU ml^{-1} was applied to the 510 subject serosurvey data, 54% of the population were considered susceptible (<0.01 IU ml^{-1}). The multiple imputation-based approach estimated that 35% of the population were susceptible. These proportions were much larger than the susceptible proportion estimated by the original ELISA measurements.

Conclusion. Testing a subset of sera by TNT combined with ROC analysis or a multiple imputation approach helps to adjust ELISA thresholds or values to assess population susceptibility more accurately. DBS is an effective low-cost alternative to serum for future serological studies for diphtheria.

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Keywords: assay validation; diphtheria; dried blood spots; enzyme-linked immunosorbent assay; multiple imputation; toxin neutralization assay.

Abbreviations: AUC, area under the receiver operating characteristic curve; CI, confidence interval; DBS, dried blood spot; IU, international unit; LMICs, low- and middle-income countries; ROC, receiver operating characteristic; TNT, toxin neutralization test.

One supplementary method and one supplementary figure are available with the online version of this article.

INTRODUCTION

Diphtheria is caused by toxin-producing strains of *Corynebacterium* species, mainly *Corynebacterium diphtheriae* and *Corynebacterium ulcerans*, and occasionally *Corynebacterium pseudotuberculosis* [1]. Diphtheria has been eliminated in high-income countries; however, it is still endemic in low- and middle-income countries (LMICs) [2]. In the last 10 years, between 4500 and 23000 cases have been reported annually worldwide [3]. Of note, over 90% of the total reported cases were concentrated in South and South-East Asia in the late 2010s [2]. However, in 2022, seven European countries reported an unexpected increase in cases, many in asylum seekers [4]. The increased number of cases among migrants in Europe and refugee camps in Bangladesh, Venezuela and Yemen highlighted the potential resurgence of diphtheria in many parts of the world [4–8].

This increasing trend of cases raises the concern that some populations have increased susceptibility to diphtheria. Insufficient vaccine coverage or an ineffective vaccine or immunization schedule can produce a susceptible population [9]. Serological surveys are potentially the most direct and informative way to determine the susceptible population [10]. Accurate measurement of antibody levels is essential to monitor population immunity and the immune status of individuals at an increased risk of infection (e.g. travellers and healthcare professionals) [11]. The gold-standard method for measuring the level of functional neutralizing antibody against diphtheria toxin in serum is the Vero cell toxin neutralization test (TNT) [12]. However, TNT is not widely used in LMICs as it is labour intensive and requires facilities for cell culture [13].

ELISAs can be a simpler, faster and lower-cost alternative to TNT, especially for a large-scale epidemiological survey [12]. Dried blood spots (DBSs) on filter paper are also a simple and low-cost method to collect serum antibodies without requiring on-site facilities for serum separation and a cold chain for transport and storage [13]. Antibodies collected on DBSs are stable at room temperature for at least a week [14, 15]. Furthermore, DBS collected by finger prick is minimally invasive and requires only a small blood volume, which is also an advantage for studies targeting young children [14]. Therefore, ELISA measurements of DBS samples could be suitable for seroepidemiological studies in LMICs. ELISAs detecting IgG against diphtheria toxoid are available that claim to report titres equivalent to TNT against native toxin. However, ELISA titres do not necessarily correlate well with TNT when titres are less than 0.1 IU ml^{-1} [11, 16]. Previous validation studies concluded that 0.1 IU ml^{-1} in ELISA could be used as a standard cut-off value to identify protected individuals. However, the correlation between the ELISA and TNT values was poor when ELISA values were in the ranges 0.01–0.1 IU ml^{-1} (some degree of protection) and <0.01 IU ml^{-1} (susceptible) [17–20].

This study tested two methods to correct ELISA anti-diphtheria toxoid titres (with serum or DBS) in order to more accurately estimate serum TNT titres, in particular to define samples in the susceptible range more reliably. The two methods were then applied to ELISA results from a recent serological survey in Vietnam to demonstrate the difference in the crude and corrected population immunity estimates.

METHODS

Sample collection

An age-stratified cross-sectional seroprevalence survey was conducted in Nha Trang City, Vietnam, in 2017. A total of 510 subjects aged 0–55 years were recruited by simple random sampling based on population census data, and serum samples were collected from the participants. The detailed survey method is reported elsewhere [21]. Of the 510 participants, 100 were randomly selected and recruited in 2019 to compare the TNT and ELISA methods. As 4 individuals were lost to follow-up, two types of specimens, serum and DBS, were collected from 96 individuals and were available for parallel comparison. The required sample size for comparing values in paired samples was justified by the sample size used in the previous study [20, 22, 23].

Written informed consent was obtained from each participant or guardian, if the participant was younger than 16 years old, when they were invited to community health centres for blood drawing. Whole blood (2 ml from participants younger than 5 years old and 5 ml from the remaining participants) was drawn by venepuncture and collected in 5 ml blood collection tubes with a clot activator (3A Medical). Whole blood was also applied to Whatman 903 protein saver cards until blood saturated a 0.5 inch (1.3 mm) diameter circle on the card and allowed to dry, following the standard sample collection and storage method for DBSs recommended by the USA Centers for Disease Control and Prevention [24, 25]. Both types of samples were transported to the Pasteur Institute in Nha Trang (Vietnam) on the day of sample collection. Whole blood samples were left at room temperature for about 30 min to allow blood samples to clot before centrifugation. Serum samples were stored in a −80 °C freezer immediately after processing. DBSs were punched out with a 6 mm hole punch, placed in microtubes and stored in a −80 °C freezer until testing.

Laboratory assays

Anti-diphtheria toxoid IgG antibody levels were measured in serum and DBS samples using a commercial diphtheria ELISA kit (IBL; RE56191) in Vietnam. For DBSs, it was estimated that 5 µl serum was absorbed in each 6 mm diameter disc of the Whatman 903 card [15, 24–28]. Each 6 mm diameter disc was added to 500 µl elution buffer to create the equivalent of a 1:100 dilution of serum. The solution was then incubated overnight at 4 °C before conducting the ELISA. Elution buffer comprised PBS containing

Table 1. Cut-off values of anti-diphtheria toxin antibodies and interpretation [17]

Anti-toxin level (IU ml^{-1})	Interpretation
<0.01	Individual is susceptible
0.01	Lowest level of circulating anti-toxin giving some degree of protection
0.01–0.09	Level of circulating anti-toxin giving some degree of protection
≥0.1	A level of circulating anti-toxin giving long-term protection

0.05% (v/v) Tween 20 and 1% (w/v) skimmed milk [27]. ELISA was performed following the manufacturer's protocol for serum and DBS samples. According to the manufacturer, the lowest detection level of the ELISA kit was 0.004 IU ml^{-1}.

Frozen sera were transported from Vietnam to the UK to determine the levels of diphtheria anti-toxin antibodies in sera by TNT at the Respiratory and Vaccine Preventable Bacteria Reference Unit, at the UK Health Security Agency, as described elsewhere [17]. The Vero cell TNT assay is based on the capacity of diphtheria toxin to cause mammalian cell death and the neutralization of this effect by diphtheria anti-toxin antibodies when present in serum specimens. Briefly, a Vero cell (ECACC no. 84113001) suspension in minimum essential medium was added to 96-well tissue culture plates along with a defined concentration of diphtheria toxin and serially diluted serum samples for testing. They were then incubated at 37 °C with 5% CO_2 and the viability of the Vero cells in each well observed microscopically 3–4 days later. The TNT is calibrated to report results in IU ml^{-1}, and the lowest quantifiable level by TNT is 0.008 IU ml^{-1}. The TNT assay includes ten twofold dilutions of test sera and generates a result of <0.008, 0.008, 0.016, 0.032, 0.064, 0.128, 0.256, 0.512, 1.024, 2.048 or ≥4.096 IU ml^{-1}. According to the current World Health Organization (WHO) laboratory manual, an individual's serum toxin neutralizing level measured by TNT is classified between susceptible and conferring long-term protection, according to its level (Table 1) [17].

Statistical analysis

Firstly, the 96 samples with both TNT and ELISA results were used to evaluate the accuracy and agreement of the two measurements in sera and DBSs. If TNT measurements of toxin-neutralizing antibody were lower than the lowest detection level (0.008 IU ml^{-1}), then 0.004 IU ml^{-1} (i.e. half of 0.008 IU ml^{-1}) was imputed for those samples following the similar method used in the previous study [16, 19]. Serum anti-toxin levels measured by TNT and ELISA were classified as ≥0.1 IU ml^{-1} (positive), 0.01–0.1 IU ml^{-1} (equivocal) and<0.01 IU ml^{-1} (negative). Two-by-two tables were created using possible combinations of two cut-off values in TNT and ELISA for two different types of specimens (i.e. serum and DBS samples). Sensitivity, specificity, area under the receiver operating characteristic (ROC) curve (AUC) and Cohen's kappa coefficients with 95% confidence intervals (CIs) were calculated [29, 30]. AUC values were classified as excellent (0.9 to 1.0), good (0.8 to <0.9), fair (0.7 to <0.8), poor (0.6 to <0.7) and failed (0.5 to <0.6) [30, 31]. The kappa coefficient was interpreted as very good (0.81–1.00), good (0.61–0.80), moderate (0.41–0.60), fair (0.21–0.40) or poor (<0.2) [32].

In addition to assessing the categorical agreement, an assessment of numeric IgG values was conducted after each value was $\log_{10}$transformed. Pearson's correlation coefficients and Lin's concordance-correlation coefficients were estimated with 95% CI to examine the association between the two measurements and the reproducibility of the test [33, 34]. A coefficient value of >0.9 was interpreted as very good, and>0.8 was interpreted as good reproducibility [16, 34]. Bland–Altman analysis was also applied to assess the agreement of TNT and ELISA values.

Secondly, the optimal ELISA cut-off values for the classification of long-term protection (equivalent to 0.1 IU ml^{-1} in TNT), some degree of protection (equivalent to 0.1–0.01 IU ml^{-1} in TNT) and susceptible (equivalent to 0.01 IU ml^{-1} in TNT) were determined by ROC curve analysis [35]. The point with maximum values of the Youden index on the ROC curve was determined as the optimal cut-off point. The sensitivity and specificity of the new optimal cut-off values were calculated to confirm their diagnostic accuracy.

Thirdly, a statistical method based on the multiple imputation approach was applied to reconstruct the distribution of nine discrete TNT values (from 0.004 to 1.024 IU ml^{-1}, excluding 2.048 IU ml^{-1} as no samples took this value and above) and estimate TNT measurement in 510 sera collected in the population-based survey in Vietnam in 2017. In this study, 96 TNT values were available in the dataset; however, TNT values for the remaining samples collected during the survey were missing. With the observed linear association between IgG values measured by ELISA and TNT in 96 reference samples, a multiple imputation generated 1000 imputed values of TNT in each sample (N=510). The 95% CIs for pooled estimates of seroprevalence based on imputed data were calculated by Rubin's rule [36]. The detailed methods are described in the supplementary material (available with the online version of this article).

Finally, the age-stratified seroprevalence in Nha Trang City, Vietnam, in 2017 was re-estimated with three different combinations of data and cut-off values: (i) using the original ELISA measurements in serum with standard cut-off values (0.1 and 0.01 IU ml^{-1}), (ii) using original ELISA measurements in serum with the optimal cut-off values determined by ROC curve analysis, and (iii)

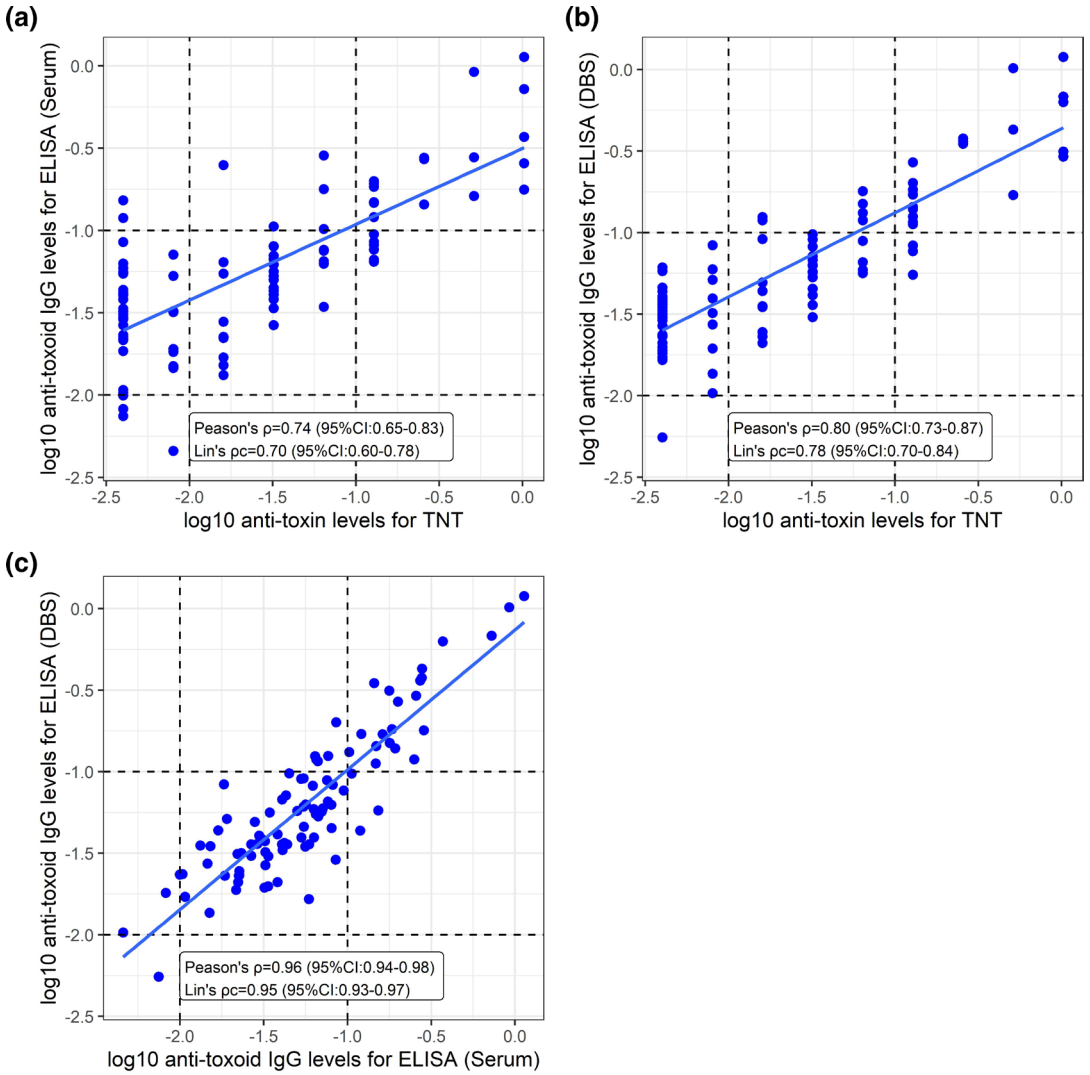

Fig. 1. Comparison of the values of TNT and ELISA in serum (a),TNT and ELISA with DBS (b), and ELISA in serum and ELISA in DBS (c) with fitted lines' equation, Pearson's correlation coefficient and Lin's concordance correlation coefficient.

using estimated TNT measurements by multiple imputation-based method with standard cut-off values (0.1 and 0.01 IU ml^{-1}). Statistical analyses were conducted using STATA15 and R software [37, 38].

RESULTS

Distribution and classification of antibody levels in 96 samples

In order to study the agreement between anti-diphtheria toxoid IgG levels in ELISA and TNT, reported titres were categorized into three classes: ≥0.1 IU ml^{-1} (positive), 0.01–0.1 IU ml^{-1} (equivocal) and <0.01 IU ml^{-1} (negative). When testing 96 samples of matched sera and DBSs, 40 sera were classified as negative by TNT, while only 4 sera and 1 DBS were classified as negative by ELISA. In contrast, 33 sera were classified as equivocal by TNT, while 68 sera and 69 DBSs were classified as equivocal by ELISA. Among 96 paired samples, about one-half of the samples with equivocal ELISA results (0.01–0.1 IU ml^{-1}) were classified as negative (<0.01 IU ml^{-1}) by TNT (Table 1). Generally, antibody levels measured by TNT were lower than those measured by ELISA, especially when TNT values were lower than 0.1 IU ml^{-1} (Fig. 1, Table 2).

Diagnostic performance of ELISA compared with TNT

Sensitivity, specificity, AUC and Cohen's kappa coefficient of ELISA against TNT results with two cut-off values of 0.1 and 0.01 IU ml^{-1} were calculated using the same 96 paired sera and DBSs (Table 3). AUC showed good performance in the ELISA

Table 2. Comparison of TNT and ELISA values in three categories: <0.01, 0.01–0.1 and ≥0.1 IU ml^{-1}

TNT (IU ml^{-1})	ELISA serum (IU ml^{-1})				TNT (IU ml^{-1})	ELISA DBS (IU ml^{-1})				ELISA serum (IU ml^{-1})	ELISA DBS (IU ml^{-1})			
	<0.01	0.01–0.1	≥0.1	Total		<0.01	0.01–0.1	≥0.1	Total		<0.01	0.01–0.1	≥0.1	Total
<0.01	4	34	2	40	**<0.01**	1	39	0	40	**<0.01**	1	3	0	4
0.01–0.1	0	28	5	33	**0.01–0.1**	0	27	6	33	**0.1–0.01**	0	63	5	68
≥0.1	0	6	17	23	**≥0.1**	0	3	20	23	**≥0.1**	0	3	21	24
Total	4	68	24	96	**Total**	1	69	26	96	**Total**	1	69	26	96

Table 3. Sensitivity, specificity, Cohen's kappa index and AUC of ELISA results with sera or DBSs compared with TNT (with sera) for different cut-offs

95% CIs are shown in parentheses.

Characteristic	TNT cut-off	ELISA cut-off	
		Serum 0.01 IU ml^{-1}	**DBS 0.01 IU ml^{-1}**
Sensitivity	0.01 IU ml^{-1}	100% (94, 100%)	100% (94, 100%)
Specificity		10% (3, 24%)	3% (0.1, 13%)
Kappa index		0.11 (0.02, 0.21)	0.03 (−0.02, 0.08)
AUC		0.55 (0.50, 0.6)	0.51 (0.49, 0.54)
		Serum 0.1 IU ml−1	**DBS 0.1 IU ml−1**
Sensitivity	0.01 IU ml^{-1}	39% (27, 53%)	46% (33, 60%)
Specificity		95% (83, 99%)	100% (91, 100%)
Kappa index		0.31 (0.15, 0.47)	0.42 (0.26, 0.58)
AUC		0.67 (0.60, 0.74)	0.73 (0.67, 0.8)
		Serum 0.1 IU ml−1	**DBS 0.1 IU ml−1**
Sensitivity	0.1 IU ml^{-1}	74% (52, 90%)	87% (66, 97%)
Specificity		91% (81, 96%)	92% (83, 97%)
Kappa index		0.63 (0.43, 0.83)	0.75 (0.55, 0.95)
AUC		0.82 (0.72, 0.92)	0.89 (0.82, 0.97)
Characteristic	**ELISA cut-off**	**ELISA cut-off**	
		DBS 0.01 IU ml^{-1}	
Sensitivity	Serum 0.01 IU ml^{-1}	100% (96, 100%)	
Specificity		25% (0.6, 81%)	
Kappa index		0.39 (0.23, 0.55)	
AUC		0.63 (0.38, 0.87)	
		DBS 0.1 IU ml−1	
Sensitivity	Serum 0.1 IU ml^{-1}	88% (68, 97%)	
Specificity		93% (85, 98%)	
Kappa index		0.78 (0.58, 0.98)	
AUC		0.90 (0.83, 0.98)	

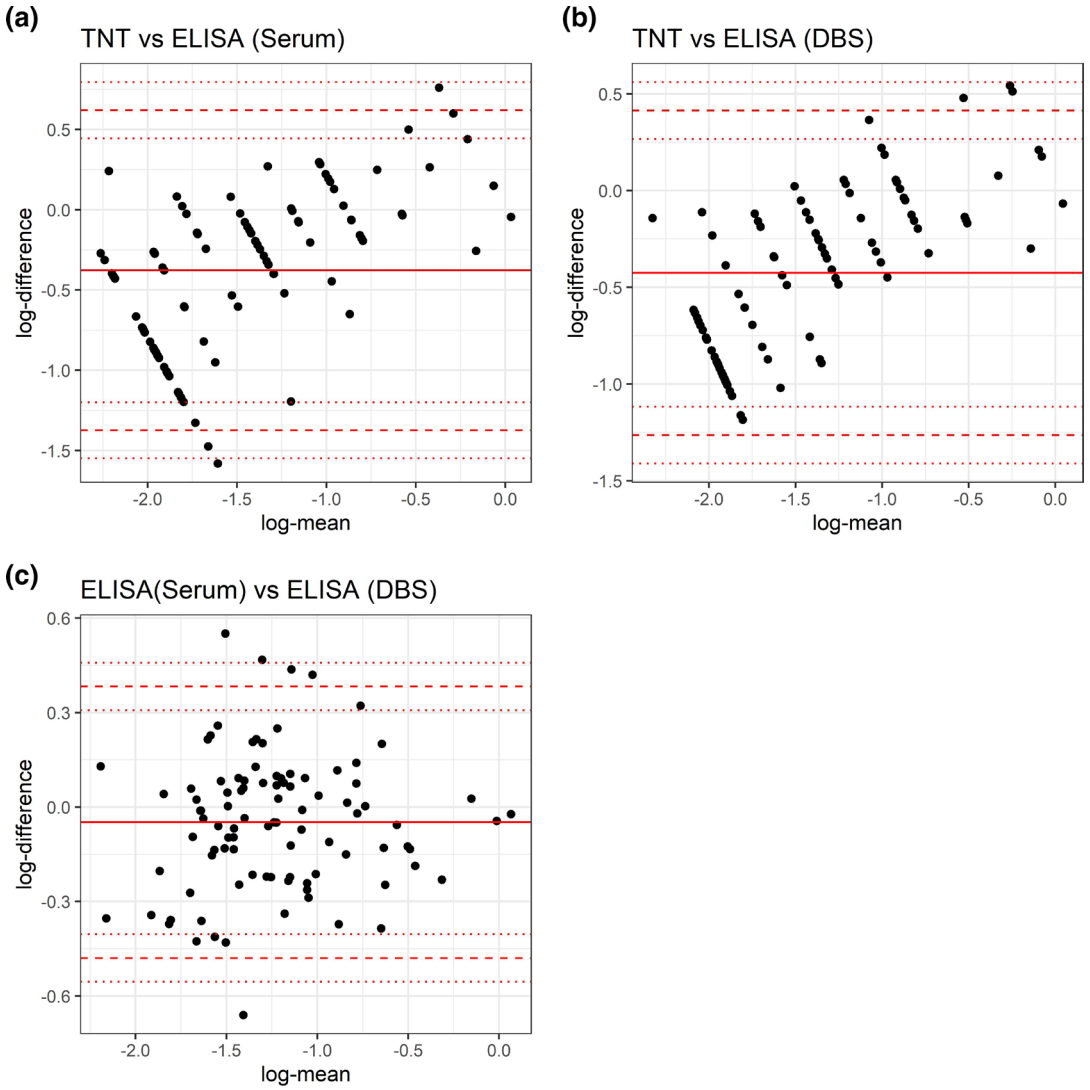

Fig. 2. Bland–Altman plots for TNT and ELISA values in serum samples (a) and DBS samples (b), and for ELISA values in serum samples and DBS samples (c). *x*-axes show the mean of paired TNT anti-toxin and ELISA anti-toxoid values; *y*-axes show the difference of paired values. Both TNT and ELISA values were transformed by $\log_{10}$. Red lines, mean log-difference; red dashed lines, 95% limits of agreement; red fine dotted lines, 95%CI of upper and lower limits of agreement.

test with a cut-off value of 0.1 IU ml^{-1} for both sample types (0.82 and 0.89 for serum and DBS, respectively), but it showed fair or poor performance with a cut-off value of 0.01 IU ml^{-1}. Similarly, Cohen's kappa showed a good agreement between the two tests with the cut-off value of 0.1 IU ml^{-1} (0.63 and 0.75 for serum and DBS, respectively); however, the agreement was fair or poor with a cut-off value of 0.01 IU ml^{-1} [32].

Correlation and concordance between TNT and ELISA values

Pearson's correlation coefficients (r) showed high correlations between TNT and ELISA values (r=0.74 in serum, r=0.80 in DBS). In contrast, Lin's concordance correlation coefficients (ρc) of ELISA against TNT were slightly below the level for good agreement for both serum and DBS samples (ρc=0.70 in serum, ρc=0.78 in DBS). The concordance between ELISA values measured in serum and DBS was very good (ρc=0.95) (Fig. 1). The Bland–Altman plots comparing TNT and ELISA samples were not symmetric even allowing for the TNT reporting discrete values. The Bland–Altman plots show that the lower the ELISA values were, the larger the difference between ELISA values and TNT values. The same trend was observed for both serum and DBS samples. The Bland–Altman plot comparing ELISA in serum and DBS samples was symmetric, although a few samples were plotted outside the 95% agreement limit (Fig. 2).

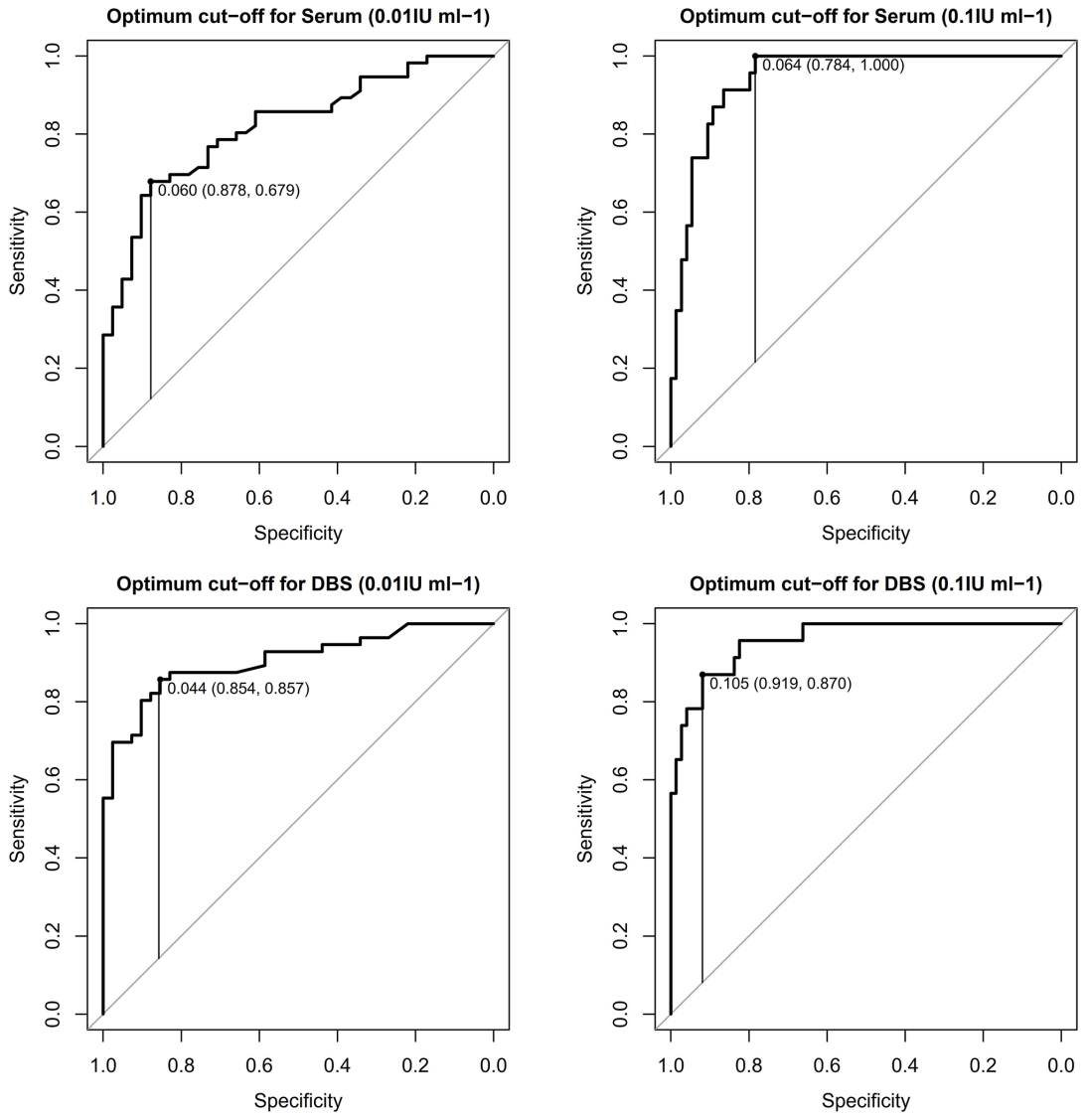

Fig. 3. Optimal cut-off values for ELISA in serum and DBS samples that classify individuals as susceptible (TNT <0.01 IU ml^{-1}) or long-term protected (TNT ≥0.1 IU ml^{-1}). Each graph shows the optimal cut-off value and its specificity and sensitivity for ELISA using serum or DBS when each optimal cut-off value classifies seropositivity. The vertical line shows the Youden index for each ROC curve.

Optimal cut-off values for ELISA identified by ROC curve analysis

The optimal cut-off values for ELISA, which classified the individuals into long-term protection (≥ 0.1 IU ml^{-1}), some degree of protection (0.01–0.09 IU ml^{-1}) and susceptible (< 0.01 IU ml^{-1}) against diphtheria (as defined by the TNT) were identified by the point with maximum Youden index on the ROC curves. For serum samples, the cut-off values of 0.060 and 0.064 IU ml^{-1} in ELISA corresponded to the cut-off values of 0.01 and 0.1 IU ml^{-1} in TNT, respectively. For DBS samples, the cut-off values of 0.044 and 0.105 IU ml^{-1} corresponded to the values 0.01 and 0.1 IU ml^{-1} in TNT, respectively (Fig. 3). The performance of the ELISA test expressed as sensitivity and specificity improved when the cut-off values were 0.060 and 0.044 IU ml^{-1} for serum and DBS, respectively, compared with 0.01 IU ml^{-1} for TNT. However, the performance of ELISA did not improve when the cut-off values were 0.064 and 0.105 IU ml^{-1} for serum and DBS, respectively, compared with 0.1 IU ml^{-1} for TNT (Table 3, Fig. 3).

Estimated proportion of susceptible individuals by new cut-off values

Using 510 serum samples collected in 2017 in a seroepidemiological study in Nha Trang City, Vietnam, the proportion of seropositive individuals based on the new optimal cut-off values for the ELISA was re-estimated using 0.064 IU ml^{-1} corresponding to a TNT value of 0.1 IU ml^{-1}, and 0.060 IU ml^{-1} corresponding to TNT value of 0.01 IU ml^{-1}. The overall estimated seropositive proportion of the population with a cut-off value of 0.064 IU ml^{-1} in Nha Trang City was 44% (95% CI 40–48 %), while it would

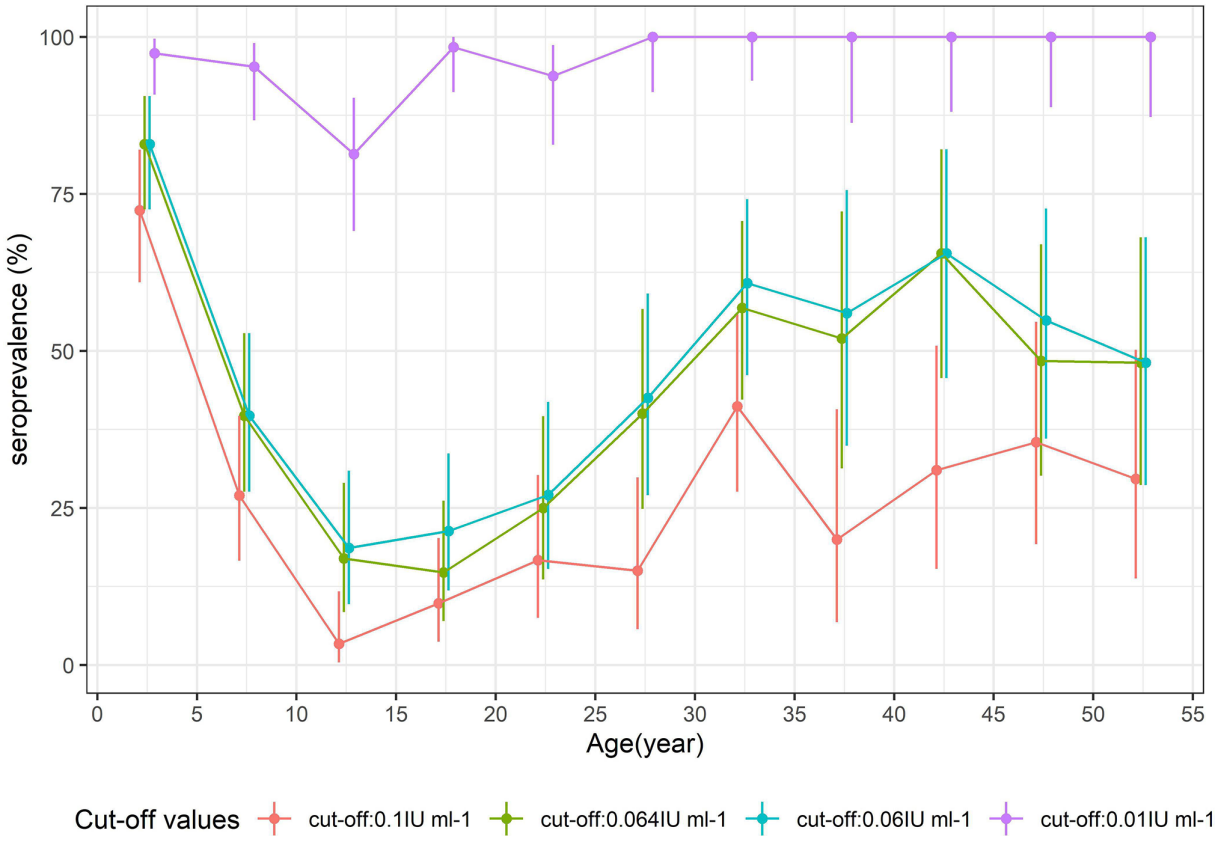

Fig. 4. Age-stratified seroprevalence for diphtheria in Nha Trang, Vietnam, in 2017, (with 95% CIs) comparing standard cut-off values, 0.1 and 0.01 IU ml^{-1}, and ROC-derived cut-off values, 0.064 and 0.06 IU ml^{-1}, in ELISA using serum samples.

be 29% (95% CI 25–33 %) with an ELISA cut-off value of 0.1 IU ml^{-1}. The estimated seropositive proportion of the population with a cut-off value of 0.060 IU ml^{-1} was 46% (95% CI 42–51 %), while it would be 96% (95% CI 94–97 %) with an ELISA cut-off value of 0.01 IU ml^{-1} (Fig. 4).

Estimated proportion of susceptible individuals by multiple imputation approach

For comparison, TNT anti-diphtheria toxin antibody levels for the 510 serum samples were estimated by applying the multiple imputation approach to ELISA data. For this, 1000 estimated anti-diphtheria toxin antibody levels in TNT were categorized into<0.01, 0.01–0.1 and ≥0.1 IU ml^{-1}. Based on this classification, overall seroprevalence and age-stratified seroprevalence by 5 year age band were calculated for each cut-off value of 0.1 and 0.01 IU ml^{-1}. The pooled estimate of seroprevalence based on the imputed data with a cut-off value of 0.1 IU ml^{-1} was 20% (95% CI 15–24 %), similar to the original data (29%). The pooled estimate of the proportion of the susceptible population (<0.01 IU ml^{-1}) was 65% (95% CI 60–70 %), which was much lower than that measured with the original ELISA data (96%) (Fig. 5). Anti-diphtheria toxoid IgG seroprevalence declined most at age 10–14 years and increased with age afterward. This immunity pattern was consistent over the three methods (Figs 4 and 5). All three analyses suggested that 0.1 IU ml^{-1} by ELISA is a good cut-off value to identify individuals with long-term protection.

DISCUSSION

This study first compared a commercial ELISA kit and TNT for measuring the anti-diphtheria toxoid antibody in serum or DBS samples. The parallel comparison was conducted using 96 paired serum and DBS samples, including samples with low titre (<0.1 IU ml^{-1}). With ELISA, more samples were categorized into 0.01–0.09 IU ml^{-1}, a 'grey zone' not included in a protective or susceptible category in TNT. The diagnostic performance of the ELISA in both serum and DBS samples was good when the cut-off value was 0.1 IU ml^{-1}, but was not adequate when the cut-off value was 0.01 IU ml^{-1}. ELISA values in serum or DBS samples correlated well with TNT values as aggregated data; however, one-to-one concordance between paired values was not good. The Bland–Altman method indicated that when ELISA values were lower than 0.1 IU ml^{-1}, TNT values were likely lower

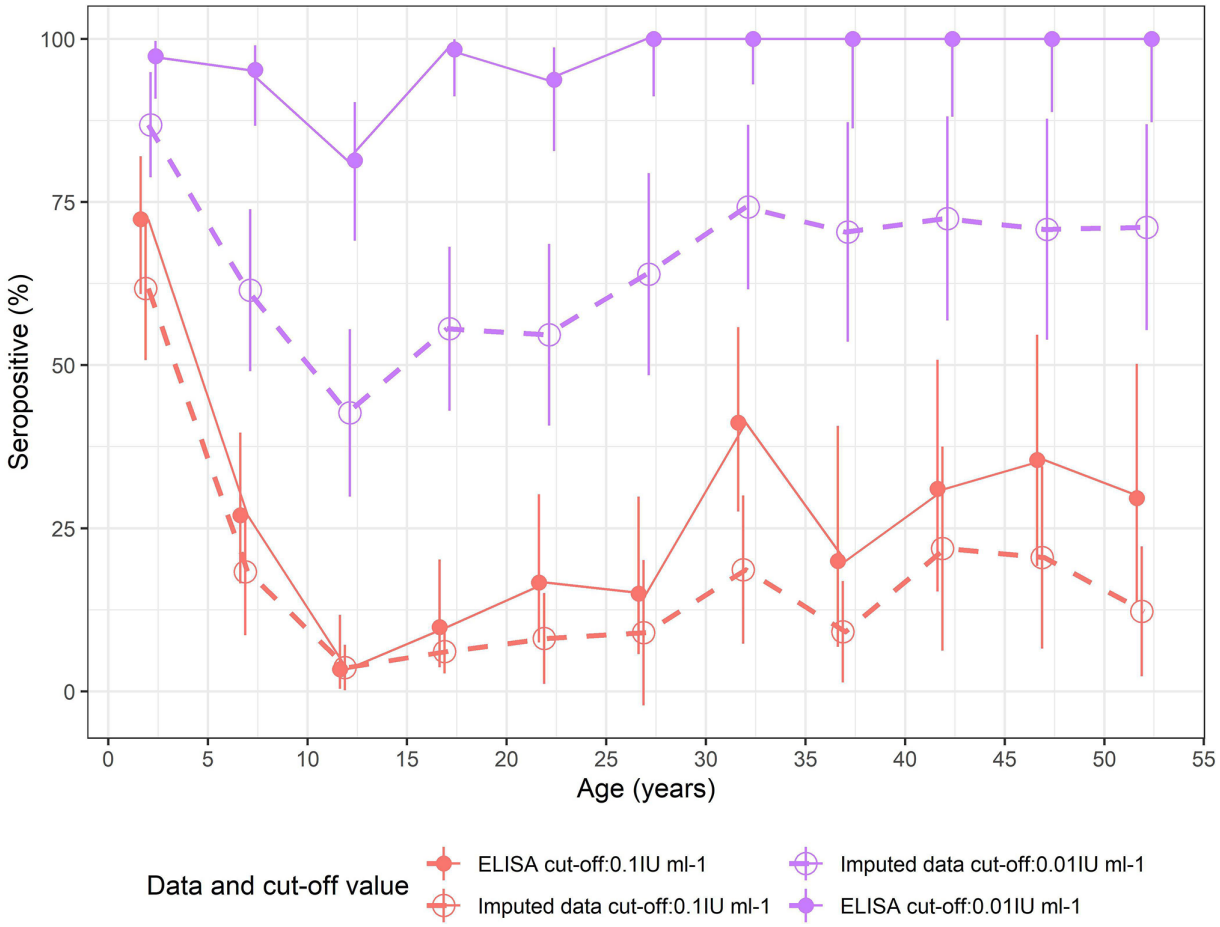

Fig. 5. Age-stratified seroprevalence for diphtheria in Nha Trang, Vietnam, in 2017, (with 95% CIs) classified by standard cut-off values (0.1 and 0.01 IU ml^{-1}): comparison of original ELISA data using serum samples and pooled estimates from imputed data. Solid lines are seroprotection levels in different age groups based on the original ELISA data. Dashed lines are pooled estimates of seroprotection levels in different age groups based on the imputed data.

than ELISA values. However, when ELISA values were greater than or equal to 0.1 IU ml^{-1}, the TNT values tended to be higher than the ELISA values.

The good linear correlation between serum TNT and ELISA anti-diphtheria toxoid results with DBSs was previously demonstrated by Schou *et al.* [22]. Our study also found that ELISA with DBS samples had better diagnostic performance than ELISA with serum samples, which might have occurred by chance due to the small sample size. Another explanation was that DBS samples requiring fewer processing steps reduced factors influencing antibody concentrations, such as blood coagulation or haemolysis, although the specific reason was not apparent. ELISA measurements in DBS and serum samples were well correlated and agreed with each other, which was also confirmed in Bland–Altman analysis. All these findings suggest that DBS is a suitable alternative to serum samples.

Due to observations that anti-diphtheria toxoid ELISA tends to overestimate TNT anti-diphtheria toxin neutralization titres, a titre of 0.1 IU ml^{-1} in ELISA is often used pragmatically as a predictor for a protective threshold corresponding to 0.01 IU ml^{-1} in TNT [39]. ROC curve analysis identified the optimal cut-off values for ELISA corresponding to two TNT thresholds. In serum samples, 0.06 and 0.064 IU ml^{-1} in ELISA corresponded to the TNT values 0.01 and 0.1 IU ml^{-1}, respectively. These two optimal cut-off values were similar. This might have occurred by chance due to the small sample size. Investigating the ROC curve in Fig. 3, another potential cut-off value for serum corresponding to TNT 0.1 IU ml^{-1} appeared to exist with a very similar Youden index. If that point were selected, the optimal cut-off value would be quite different. In DBS samples, 0.044 and 0.105 IU ml^{-1} in ELISA were equivalent to TNT values of 0.01 and 0.1 IU ml^{-1}, respectively. The optimal cut-off threshold of long-term protection in DBS samples, 0.105 IU ml^{-1}, was nearly equal to the standard threshold of 0.1 IU ml^{-1} in TNT. This result also suggested that the ELISA performed on DBS samples could provide a proxy measure of TNT when using a cut-off value of 0.1 IU ml^{-1}. If the same ELISA kit is used, these cut-off thresholds could be potentially used to measure seroprevalence in a population where the

TNT assay is unavailable. Although ROC analysis is a common method to identify the optimal cut-off points for screening tests, our study is the first study, to the best of our knowledge, that has applied ROC analysis to identify new optimal cut-off thresholds for anti-diphtheria toxoid antibodies. One limitation of our study is that it used only one commercial ELISA kit. As each ELISA kit produces titres with a different level of correlation with TNT [11, 17], the optimal cut-off values would need to be similarly calculated for each commercial ELISA kit.

The final goal of this study was to estimate the population's susceptibility to diphtheria more accurately based on available ELISA results. While ROC analysis converted the continuous ELISA values into the binary categories of TNT, another approach estimated each individual's TNT measurement based on the linear association between continuous values of ELISA and TNT measurements. A previous study used linear or quadratic regression models to predict TNT measurements from the ELISA measurements [19]. In contrast, our study applied a multiple imputation approach based on linear regression. This approach considers the distribution of samples and took a pooled mean of 1000 estimates considering the uncertainty of the association between ELISA and TNT; therefore, it could provide better estimates compared with simple linear regression. The pooled seroprevalence estimates suggested that about one-third of the population was susceptible to diphtheria in Nha Trang City, which was much higher than the estimates based on the original ELISA results. Small-scale diphtheria outbreaks continue to occur in Vietnam, which suggests that susceptible individuals remain in the population [40, 41]. Therefore, the pooled seroprevalence estimates might have reflected the Vietnamese population immunity more accurately, although the real seroprevalence was not measured by TNT. If resources allow measurement of the antibody levels in the 510 samples by TNT, we will be able to estimate the actual seroprevalence based on TNT in this study population and verify our results directly. The multiple imputation-based approach required only a proportion of samples to be tested by TNT to estimate the TNT measurement for all survey participants who only had ELISA results. This method could be considered to estimate seroprevalence for future large epidemiological studies.

One of the limitations of this study was that the TNT and ELISA measurements were not duplicated to reduce measurement errors, although both TNT and ELISA methods contain quantitative internal controls. The 96 reference samples used for parallel comparison between TNT and ELISA had a skewed distribution towards low concentration of IgG; 40 (42%) of the samples were <0.01 IU ml^{-1} and 73 (76%) were <0.1 IU ml^{-1} measured by TNT (Table 1). Although the samples with low values were ideal for addressing the challenge of working with this ELISA, the results may differ in other datasets with different distributions. For negative TNT results, 0.004 IU ml^{-1} was arbitrarily used as a lower value, although the actual antibody levels might have varied. The analysis was conducted using a relatively small sample size, and the results would be more robust if repeated with a larger sample size.

Walory *et al.* measured the correlation coefficient between ELISA and other serological methods, such as the passive haemagglutination assay (PHA) and latex agglutination test (LA), with TNT. The correlation between ELISA and TNT was similar to our study (r=0.8), which was better than the other old serological techniques, while more recently developed toxin binding inhibition ELISA (ToBI) had better correlation than ELISA [20]. However, the procedure for ToBI is complicated [20]. Therefore, ELISA is likely to remain the most popular method for serological surveys for diphtheria until simpler methods are developed.

This study suggests that DBS could be a simple and low-cost alternative to serum samples to detect anti-diphtheria toxoid IgG using ELISA. A 0.1 IU ml^{-1} cut-off value in ELISA reliably identified individuals with long-term protection against diphtheria compared with TNT, especially using DBS samples. A cut-off value of 0.01 IU ml^{-1} in ELISA appears to underestimate the proportion of the susceptible population. In diphtheria serological surveys, testing a subset of samples via TNT could improve the assessment of the susceptibility against diphtheria at the population level, when combined with ROC analysis or a multiple imputation approach.

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Author contributions
N.K.: conceptualization, formal analysis, methodology, writing – original draft. A. Endo: formal analysis, methodology, writing – review and editing. L.T.L.: methodology, data curation. T.B.N.: methodology, resources. H.T.D.: resources, project administration. M.T.: writing – review and editing. L.-M.Y.: supervision. Y.M. and S.R.: methodology, data curation. A. Efstratiou: methodology, resources. N.K.F.: methodology, resources, writing – review and editing. D.L.: methodology, resources, writing – review and editing.

Conflicts of interest
A.E. received a research grant from Taisho Pharmaceutical Co., Ltd., for research outside this study.

Ethical statement

Ethical approval was obtained from the Vietnamese Ministry of Health and the London School of Hygiene and Tropical Medicine (LSHTM) ethical review boards (IRB-VN01057-27/2015, LSHTM ethics reference no. 17518/17913).

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