Understanding factors contributing to outbreaks of diphtheria and implications for vaccination policy in Vietnam

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Declaration by candidate

I, Noriko Kitamura, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: 

Date: March 28, 2023

Abstract

Background: Diphtheria is a severe, acute infectious disease caused by toxin-producing *Corynebacterium* species, mainly *C. diphtheriae*. The diphtheria toxoid vaccine successfully reduced global diphtheria incidence. However, diphtheria remains endemic in many countries. Currently, the World Health Organization recommends three primary doses during infancy and three booster doses until the adolescent period; however, many low- and middle-income countries have not introduced all booster doses. Vietnam experienced several outbreaks of diphtheria in the last decade. This thesis aims to elucidate the mechanism of diphtheria outbreaks and appropriate vaccination strategies in Vietnam.

Methods: This thesis consists of five components: first, the diphtheria outbreak in Vietnam is described with the available data (Chapter 3); second, a systematic review was conducted with age-specific seroprevalence data from 15 countries to estimate the optimal booster dose interval (Chapter 4); third, a cross-sectional and cohort study was conducted in a well-vaccinated community in Vietnam with no reported cases to assess population immunity and the waning of vaccine-derived immunity (Chapter 5); fourth, another cross-sectional carriage prevalence and seroprevalence survey was conducted in an epidemic-prone area (Chapter 6); and finally, a validation study for enzyme-linked immunosorbent assay (ELISA) was conducted via parallel comparison of ELISA and neutralising test measurements (Chapter 7).

Results: In Chapter 3, we found that 73% of diphtheria cases reported in Central Vietnam between 2015 and 2018 were in school-age children. While this finding indicated that there is an immunity gap in school aged children, Chapter 5 confirmed the low seroprevalence in the age group of 6-15 years (7%). In Chapter 3, we identified two fatal cases (7 and 13 years old) who had received three or more doses of the diphtheria-tetanus-pertussis (DTP) vaccine, indicating that vaccine-derived immunity waned or vaccine was not effective. The findings in Chapter 5 suggested that the duration of protection of vaccine-derived immunity was 4.3 years after four doses of DTP, which was much shorter than the commonly perceive 10 years. In contrast, the systematic review in Chapter 4 suggested that the interval between the fourth and fifth doses could be up to 10.3 years.

In Chapter 3, strains of the same genetic type were shared by all epidemiologically linked cases; however, it was often impossible to track the transmission chains. The findings indicated that local transmission of *C. diphtheriae* was attributed to multiple strains with asymptomatic carriers. In Chapter 6, we identified that 1.4% of the population were asymptomatic carriers; the highest carriage prevalence was observed in individuals aged 1–

5 years (4.5%), which was much higher than the recently reported carriage prevalence in Europe. Furthermore, 67% of carriers harboured a non-toxigenic strain.

Seroprevalence identified in epidemic and non-epidemic settings varied. Seroprevalence among 1–5-year-old in the epidemic-prone area was low due to the limited vaccination history and low seroconversion rate, probably derived from the children's poor nutrition status. These children (asymptomatic carriers) might maintain transmission of *C. diphtheriae* in their communities. When the bacteria reaches susceptible hosts, likely school-age children, they are detected as symptomatic cases. This is likely the mechanism of the current diphtheria outbreak in Vietnam.

Chapter 7 confirmed that the ELISA method used for the study showed appropriate protection levels in the population when a cut-off value of 0.1 IU/ml was used.

Conclusions: The most susceptible age group in Vietnam was school-age children due to the waning of vaccine-derived immunity. In addition, the recent diphtheria epidemic in Vietnam might be attributed to the low vaccine coverage due to limited healthcare access and the low seroconversion rate due to child malnutrition. Based on these findings, it was concluded that improved DTP3 coverage and a school-entry booster dose are essential to control the transmission of *C. diphtheriae* in Vietnam. In the long term, multiple booster doses will be required to reduce the susceptible population.

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Table of Abbreviations

ADPR:	adenosine diphosphate ribose
DAT:	diphtheria antitoxin
DBS:	dried blood spot
DTP:	diphtheria-tetanus-pertussis vaccine
DTP3:	vaccination coverage of those who received three primary-dose series of DTP
dtx:	diphtheria toxin genes
DtxR:	diphtheria toxin repressor protein
dtxR:	diphtheria toxin repressor gene
DHS:	demographic health survey
EF-2:	elongation factor 2
ELISA:	enzyme-linked immunosorbent assay
EPI:	expanded programme on immunization
GMC:	geometric mean of concentration
Hib:	*Haemophilus Influenzae* b vaccine
HepB:	hepatitis B vaccine
LMICs:	low- and middle-income countries
MICS:	multiple indicator cluster survey
MMR:	measles-mumps-rubella vaccine
MoH:	Ministry of Health
MALDI-TOF:	matrix-assisted laser desorption ionization-time-of-flight mass spectrometry
MEE:	multi-locus enzyme electrophoresis
MLST:	multi-locus sequence typing
MUAC:	mid upper arm circumference
NAD:	nicotinamide adenine dinucleotide
NGS:	next generation sequencing

NTTB: non-toxigenic *tox* gene-bearing

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

ROC receiver operating characteristic

SAE: severe adverse event

SIA: supplemental immunisation activity

TNT: toxin neutralisation test

WGS: whole-genome sequence

WHO: the World Health Organization

WUENIC: World Health Organization (WHO)–United Nations Children's Fund (UNICEF) estimates of national immunization coverage

Chapter 1: Introduction

1. General introduction

Diphtheria is a disease caused by toxigenic *Corynebacterium diphtheriae, Corynebacterium ulcerans,* and *Corynebacterium pseudotuberculosis* and, mainly affects the upper respiratory tract. It has largely been controlled by the diphtheria toxoid vaccine; however diphtheria remains endemic in many parts of the world. Since 2010, several large-scale outbreaks have been reported across the globe. Non-toxigenic *C. diphtheriae* is emerging in the United Kingdom and Europe, where diphtheria had been eliminated. Diphtheria is being recognised as a re-emerging global disease. Diphtheria is one of the most well-studied diseases in the history of bacteriology; however, many characteristics of this bacteria remain unknown.

1.1. Global disease burden of diphtheria

Diphtheria was a major cause of child death in the early 20th century, especially in temperate zones. However, the number of reported cases of diphtheria declined sharply after the introduction of the toxoid vaccine. The diphtheria toxoid vaccine was first produced by Ramon in 1923 (1). It began to be used widely in North America (the United States and Canada) in the 1920s and in Western Europe between the 1940s and the 1950s (2). In Canada, the diphtheria incidence rate was 98 per 100,000 population in 1924 and declined to ~0 per 100,000 by 1969 following the introduction of the vaccine in 1926 (3). In England and Wales, the annual incidence of diphtheria in 1940 exceeded 61,000, with 3,283 deaths, and it declined to 38 cases and six deaths in 1947 after the introduction of the vaccine in 1941 (4).

In many low- and middle-income countries (LMICs), the diphtheria toxoid-containing vaccine was introduced at the time of initiation of the Expanded Programme on Immunization (EPI) in 1974. In 1980, the annual global incidence was about 100,000, according to the World Health Organization (WHO), and it declined rapidly to 10,000 by 2010 (5). However, in the last decade, multiple outbreaks have been observed in South Africa (6), Nigeria (7), Madagascar (8), Yemen (9), India, Indonesia (10, 11), Thailand (12, 13), Lao PDR (14, 15), the Philippines (16), Vietnam (17-19), the Bangladesh-Myanmar border (20-22), Brazil (23, 24), Colombia (25), Haiti (26), and Venezuela (27, 28). The largest outbreak in the 21st century occurred in a refugee camp at the Bangladesh-Myanmar border (Chapter 1 Table 1) (20). Over 7,000 probable cases were reported among Rohingya refugees, and over 5,000 cases were identified in each of Yemen and Venezuela.

The form of the diphtheria toxoid vaccine has been changing since its development (29, 30). Today, the diphtheria-tetanus-pertussis (DTP) vaccine is mainly given to children combined with the hepatitis B and *Haemophilus Influenzae* type B vaccine in LMICs as a pentavalent

vaccine (DTP-Hib-HepB). Until the early 1940s, only a single-dose vaccine was used for immunisation (31); however, it was found that single-dose vaccination protected only 5% of recipients (32). Thereafter, a two-dose schedule at 6–12 months with a 5-month interval was thought to provide adequate protection (33, 34). By 1980, the current three-dose primary schedule was fixed, as three doses at more than 3-week intervals was confirmed to protect 96% of children before school entry (35). Antibody concentration and affinity will increase until four doses of vaccine are provided in the first 2 years of life; however, more than four doses does not increase the duration of protection and is not recommended for children under 1 year old (36). In the late 1980s, an accelerated schedule that starts at 6 weeks of age with a 1-month interval was introduced to increase the immunisation opportunities for children in LMICs. However, the immunity levels of 1-year-olds who received three doses in an accelerated schedule were not as high as in a wide-spaced schedule until a booster dose was given in the second year of life (37).

Clinical trials for measuring vaccine efficacy have never been conducted for a diphtheria toxoid vaccine because the massive reduction in morbidity and mortality after its introduction clearly demonstrated its effectiveness (38). A case-control study conducted in the former Soviet Union in the 1990s showed relatively high vaccine effectiveness for one dose (78–93%) and for two doses (85–100%) among children younger than 14 years of age (39-41). The result appeared to be controversial against the current WHO recommendation of DTP booster doses in the second year of life (18 months old), at school entry (4–7 years old), and school leaving (9–15 years old) (38). According to the most recent report based on the systematic review from the WHO, vaccine-derived anti-diphtheria antibodies are maintained above protection levels for more than 10 years (42).

1.2. Common risk factors contributing to outbreaks during the immunisation era

Investigation of the massive epidemic in the 1990s in the former Soviet Union, which reported more than 150,000 cases and 4,500 deaths, suggested four favourable underlying conditions for infection transmission (43, 44):

- living under crowded and suboptimal hygienic conditions
- decreased infant vaccination coverage
- increasing susceptibility in adults after the successful child vaccination program, and
- increased travel and mass population movement

The same risk factors were identified in recent outbreaks. The crowded and suboptimal hygiene in refugee camps and the large number of displaced populations in Bangladesh, Yemen, and Venezuela most likely increased the transmission of infection. Additional risk factors have been reported in the literature. Attending boarding school was reported to be a

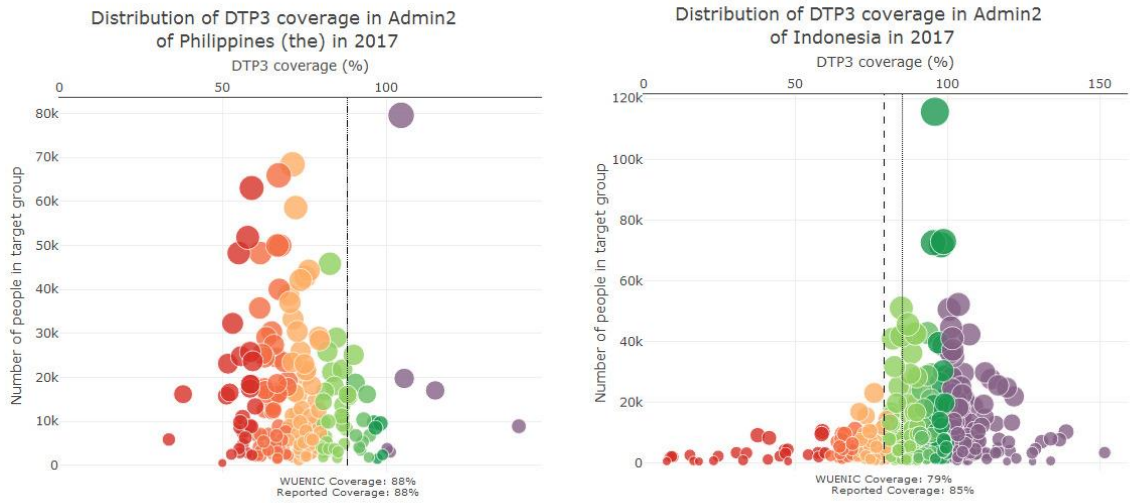
risk factor for the transmission of diphtheria in Indonesia (19, 45). The movement of children was also reported to be a factor for enhancing transmission in Indonesia (45). Poor nutrition status was reported to be a risk factor for *C. diphtheriae* infection in some studies; however, others denied this association (45, 46). Sharing beds or utensils and poor personal hygiene, such as bathing less than once a day, were reported to be risk factors in Georgia (47) and Vietnam (17). These findings indicate the correlation between skin hygiene and respiratory diphtheria infection which has been repeatedly reported in historical literature. Close person-to-person contact in the household increases the chance of droplet transmission and transmission through direct skin contact or fomites. Therefore, close contact is an important component of the transmission of *C. diphtheriae*, and it has appeared unchanged since the pre-vaccination era.

Low infant vaccination coverage is probably the most critical factor contributing to diphtheria outbreaks. Infant immunisation coverage had declined to 18–59% in urban areas of the former Soviet Union between the late 1980s and the early 1990s, immediately before large outbreaks emerged (48). Conflict and/or social instability disrupted routine infant immunisation in Yemen, Myanmar, and Venezuela (49-51). The Philippines has faced numerous diphtheria and other vaccine-preventable disease outbreaks in the last decade, which indicates that the current vaccine coverage is insufficient for disease control (52). Mistrust in vaccines was spread after discussion around the dengue vaccine in the Philippines (52). Indonesia successfully controlled the number of diphtheria cases at a low level until 1998 by promoting a massive vaccination program; however, the disease re-emerged in 2009 (53). Due to vaccine hesitancy in Indonesia, DTP3 coverage (coverage of those who received three primary-dose series of DTP) stagnated at 75.6% in 2015 (53). A negative correlation between infant vaccine coverage and diphtheria incidence was observed (54-56). In Yemen, a high diphtheria incidence was observed in areas where DTP3 coverage was low (54, 55). In Peru, diphtheria cases were newly identified after 20 years of absence of diphtheria in an area with low vaccination coverage (56). Simultaneous outbreaks of measles and diphtheria in Venezuela, Yemen, and the Philippines indicated that the low infant vaccination coverage would trigger transmissions. However, the sudden decline of coverage alone may not explain all the aspects of the recent outbreaks.

Infant vaccination coverage in Lao PDR has improved in recent decades. However, Lao PDR still occasionally reports diphtheria outbreaks, which is probably because the immunisation coverage in the community has not been adequate to eliminate toxigenic strains in this country. India accounts for the highest number of diphtheria cases in the world. India has reported several areas of low DTP3 coverage in the country, while the national average of DTP3 coverage appears high (57). The localised areas with low DTP3 coverage

could be sufficient to maintain toxigenic strains circulating in the human population or environment. Chapter 1 Figure 1 shows an example of the wide-range of DTP3 coverage at subnational levels in the country where cases were recently reported (58).

Chapter 1 Figure1. Distribution of DTP3 coverage in administrative level 2 (often called district) of Philippines and Indonesia in 2017



WUENIC: WHO-UNICEF estimates of national immunization coverage

solid line: reported coverage, dashed line: WUENIC coverage, circle size: population density in area of administrative level 2 in each country

Most of the countries that faced a recent outbreak have established one or two booster doses; however, no countries have introduced adult booster doses. Susceptibility in adults was discussed as one of the factors influencing outbreaks; however, outbreaks would not become large when susceptibility is limited only to adults. When several factors, including low infant vaccination coverage, susceptible adults, close contact in crowded housing, and population movement, are combined, outbreaks appear to become larger and spread in to extended areas.

1.3. Age shift of diphtheria cases and immunity in the population

The immunity against diphtheria toxin in the population changed after the introduction of vaccination for infants. Galazka et al. reported that the lowest immunity level was observed around aged 10–20 years in the 1970s in Poland after 10 years, the lowest immunity level was shifted to the individuals aged 30–40 years ten years later in the same country (59). In Nigeria, 85–90% of the population aged 15–40 years were immune to diphtheria in 1980, and the proportion decreased to 70% in the same age group in 2010 after the introduction of

the DTP vaccine (60). This may be because of the decrease in opportunities for natural exposure leads to the loss of immunity in individuals after the introduction of the vaccine.

The affected age group of cases has changed over time since vaccine introduction. In the pre-vaccination era, it was reported that 40% of patients were children younger than 5 years, and 70% were younger than 15 years in North America and Europe (61). This proportion changed over time. For example, in the Netherlands, 6% of cases were in adults over 18 years old in 1930, and this proportion increased to 37% in 1944 (61). In Thailand, 49% of the reported diphtheria cases were in children younger than 5 years old in the 1980s, and the proportion declined to 38% in the 1990s. In contrast, the proportion of cases aged 5–15 years old and over 15 years old increased in the 1990s compared with the 1980s from 48.8% to 53.5% and from 2.2% to 6.6%, respectively (62).

Clarke et al. analysed the age of the diphtheria cases using the case-based information reported to the WHO and found that the most affected age group of diphtheria was associated with the local DTP3 coverage (57); where the DTP3 coverage increased, the proportion of cases in individuals aged 15 years or older increased. In the recent diphtheria outbreak, mainly the 5–15 year age group was affected by diphtheria, which was different from the previous large outbreak in the former Soviet Union, in which adults comprised two-thirds of the cases (63). According to reports from Vietnam, the Rohingya population, South Africa, Nigeria, Yemen, and Indonesia, between 44% and 73% of cases were in individuals aged 5–15 years, and a wide range of ages (7 months to 70 years) was affected by diphtheria (6, 7, 9, 10, 19, 21, 22, 54).

Literature on diphtheria case reports or outbreak reports published after 2010 were identified in Embase and reviewed. Articles were reported from all over the world, but the case reports from LMICs were selected in this review. The characteristics of the recent outbreaks in 52 articles, including location, age, vaccination history, case fatality ratio (CFR), vaccination coverage, and vaccine schedule, if available, are summarised in Chapter 1 Table 1. The most affected age group was 5–15 years and cases were often not vaccinated adequately.

Chapter 1 Table 1. Reported cases, age distribution, case fatality ratio, and vaccination history in literature published between 2010-2021

Country	Area	Year	Confirmed case (reported case)	CFR (%)	Case <5yr (%)	Case <15yr (%)	Age range	DTP			Social factor	Ref
								Vaccination history	Coverage (%)	Schedule		
South Asia												
India	Gujarat	2005-2011	1,461							6,10,14w, 16-24m, and 5-6 yr		(64)
India	Assam	2009	13	30.8%	0%	30.8%	5-45y	31% full, 10% partial	62.2%			(65)
India	North Bengal Medical College	2008-2012	33	27.3%	79%	90.8% (<19y)						(66)
India	Hyderabad	2008-2012	2,925		16%	47% (<20y)		77% none				(67)
India	Lucknow region	2009-2011	279	48%	49.5%	50.5%		18% partial 80% none				(68)
India	Beliaghata, Kolkata	2009-2011	200	2.5%				75% full			low socioeconomic status	(69)
India	Agra	2009-2011	115		61.7%							(70)
India	BIjapur district, Karnataka	2011	6									(71)
India	Dhule, Maharashtra	2011	11		20%	100%						(72)
India	Jaipur, Rajasthan	2011-2014	180	24.4%	48%	98%	0-20y	19% full, 21% partial, 54% none				(73)

India	Delhi	2012-2014	218 (941)		58.4%				DTP3 study population 58-72%			(74)
India	Vijayapura district, Karnataka	2012-2015	26 (255)	2%			0-18y					(75)
India	Vijayapura district, Karnataka	2012-2015	38 (432)		55%	100%	1-15y					(76)
India	Bangalore	2015	31		26%				National DTP3 80% in 2015–6			(77)
India	North Kerala	2016	533		7%	55% (<18yr)		12% full				(78)
India	Dibrugarh, Assam	2015-2016	10		0%	30%		40% partial, 60% unknown				(79, 80)
India	6 regions	2015-2018	32 (431)									(81)
India	Telangana	2017	124	15%				53% full, 36% 5 doses				(82)
India	BLDE university Vijayapura	2018	11	27.3%				18% full, 73% partial, 9% none				(83)
India	Tamilnadu	2018-2019	5 (21)	20%		100%	<12y					(84)
Pakistan	Peshawar	2016-2017	56	8.9%						6,10,14 w		(85)
Southeast Asia												
Rohingya (Myanmar)	Myanmar-Bangladesh border	2017-2019	285 (7064)	8-31%	24.5% (<7yr)	71.3%			~30%	6,10,14 w	Conflict 0.7 million	(86)

Rohingya (Myanmar)	Myanmar-Bangladesh border	2017-2019	(8487)		13%	67%					population moved	(22)
Indonesia	Entire country	2011-2016	3,353	3.30%		69%		11.7% full, 49.3% partial, 39% none	75%	2,3,4m, 5-6y, 15y	Vaccine hesitancy, Lack of access	(10)
Indonesia	Entire country	2017	596 clinical cases	5.03% (0-20%)					National DTP3 75.6% DTP3 in the lowest district 52.9%			(53)
Indonesia	Jakarta and Tangerang	2017-2018	304	3.50%	28.6%	84.8%	1-18 y	15% full				(87)
Thailand	Entire country	1980s	6211		49%	97.8%				2,4,6 m, 1.5y, 5y, 12y		(62)
Thailand	Entire country	1990s	425	19.5%	38%	93.4%						(62)
Thailand		2010	77 per year									(13)
Thailand		2012	38	5.2%			5-72y					(12)
Lao PDR	Entire country	2012-2013	62 (168)	15-19%		69%	3m-43 y	8% full, 34% none, 56 unknown	National DTP3 67%~	6,10,14 w		(88)
Philppines	Manila	2006-2017	267	43.8%	31.1%	86.6%		47.6% full		6,10,14w, 5-6 y, 11-12 y	Vaccine hesitancy after Dengvaxia	(16)
Vietnam	HCMC/South region	1999-2004	90 (401)							2,3,4 m		(17)
Vietnam	Gia Lai	2013-2014	108	-	-	73%	1-60y	79% full	50% (2006-7),	2,3,4 m, 18m		(18)

									76% (2010-4)		DTP suspended in 2013	
Vietnam	Central Region	2015-2018	22 (46)	24%	12%	67%	1-27y		57% (local)			(19)
Malaysia	Nationwide	2016	31	10%	33%	70%	11m-41y	3% full, 9% partial		2,3,5m, 18m, 7y , 15y		(89)
South America and the Caribbean												
Brazil	Maranhao	2015	27 (57)	11%	48% (7yr)	96%		37% full, 59% partial		2,4,6m 15m, 4y		(90)
Peru	Loreto		3							2,4,6m 18m, 4y, 10y		(91)
Haiti	Entire country	2014-2017	113	14-50%				88% none or unknown		6,10,14 w	Earthquake and social disruption in 2010	(92)
Haiti	Entire country	2014-2018	189 (456)	21%		96% (<18y)		80% none				(26)
Haiti	Hopital Sacre Coeur, Northern Haiti	2015-2018	26	50%			2-15 y					(93)
Dominical Republic	Entire country	2004	80 (122)	32.5%	68.8%	100%	3m-13y	12.8% full		6,10,14 w		(94)
Venezuela	Entire country	2016-2018	1,249 (2,170)	22%					National DTP3 ~84% in 2016, 66% in 2017, <50% in 2018	2,4,6m, 18m, 5y, 10y,	Social instability 3.4 million moved	(95)
Venezuela	Entire country	2016-2019	1,559 (3,033)	13-20%	22%	69%						(49, 96)
Venezuela	Amerindian, Wonken	2016-2017	10	20%~	20%~	70%~	4-31y					(27)
Venezuela	Caracas	2019	37	16.20%			1-66y	23% full				(97)
Africa												

Nigeria	Lagos, Benin, Katsina state	2007-2008	4, 5, 10	50%, 40%, 80%					National DTP3 34.9%	6,10,14 w	Vaccine hesitancy	(60)
Nigeria	Borno state	2011	107	22.4%								(60)
South Africa	KwaZulu-Natal province	2015	15	27%	6.7%	73%	4-41y	45% full	Provincial DTP3 96%, DTP4 84%	6,10,14 w, 5-6 y		(6, 98)
Madagascar	Mahajanga	2017	1						National DTP3 86%	6,10,14 w		(8)
Other location												
Yemen		2017-2018	2,243	5-22%	18%	62%				6,10,14 w	Conflict	(9)
Yemen		2017-2020	5,701	5.8%	15%	60%		>54% full or partial	National DTP3 <80%	6,10,14 w		(55)

1.4. Bacterial carriage in endemic and eliminated area

Once an outbreak occurs, the outbreak strain remains circulating in the same area for an extended amount of time. One study examined *C. diphtheriae* isolates identified between 1973 and 1996 in the US. The study revealed that the endemic foci of toxigenic *C. diphtheriae* might have persisted in the US for more than 25 years (99). Similar findings were reported from Ontario, Canada (100). Russia has never been totally free of diphtheria after a large outbreak in the 1990s, and strains with the same ribotype as the epidemic strain had been prevalent for several years (101). Latvia continues to report a high incidence rate of diphtheria, and the respiratory carriage rate is also higher than in other parts of Europe after 20 years of diphtheria resurgence (102).

Since 1990, *C. ulcerans* has become dominant among isolates from humans in the UK and Europe (103). *C. ulcerans* has been identified in subjects with no recent travel history to endemic areas, but they often have history of contact with domestic animals. The increasing incidence of *C. ulcerans* may be associated with different pathogenicity and expanded host reservoirs (104). Moreover, non-toxigenic *C. diphtheriae* has been increasingly reported in the UK since the 1990s (105). More recently, non-toxigenic toxin-bearing (NTTB) strains were identified in clinical cases in Europe, and it has been suspected that vaccines will not protect individuals from these strains (106, 107). The increasing incidence of NTTB strains might be due to selective pressure on the toxigenic strains by the vaccine; however, the prevalence and role of non-toxigenic strains in healthy individuals as a part of the normal flora in the upper respiratory tract is poorly understood (108).

C. diphtheriae has been occasionally identified in Europe among travellers returning from endemic areas: skin lesions caused by *C. diphtheriae* have also been identified among these travellers (109, 110). The *C. diphtheriae* strain identified in travellers returning from endemic areas was ‘classical’ toxigenic *C. diphtheriae* (111), which indicates that *C. diphtheriae* is still dominant in endemic areas. Therefore, the causal pathogen in recent outbreaks at LMICs is classical *C. diphtheriae,* not the NTTB strain or *C. ulcerans*. However, there are no data on the carriage prevalence of *C. diphtheriae*, and other species in LMICs, especially the non-toxigenic strain, have not been isolated in the resource-limited settings as intensive laboratory resources are required to identify.

The carriage prevalence of both toxigenic and non-toxigenic bacteria was measured in the UK in the 1970s and reported to be between 0.5% and 1.2% in the non-epidemic phase and increased to 25–40% when an outbreak occurred (112). In the 2000s, the carriage prevalence was reported to be 0.05–0.07% among children in Italy and Greece. The highest carriage prevalence was 0.37% in Latvia among entire population, where diphtheria cases

have been continuously reported since the 1990s (113). Chapter 1 Table 2 summarises the respiratory carriage prevalence in different populations and age groups. Carriage prevalence among contacts of the cases (No.4 to 7) was higher than in the general population (114-118).

Chapter 1 Table 2. Respiratory carriage prevalence of *C. diphtheriae* in different countries and years

No	Respiratory Carriage	Country	Year	Population	Proportion Non-toxigenic	Sample	Ref
1	8.4% [6.1%-11%]	Nigeria	1961	1-10 yr child (N=500)	82%	Throat	(119)
2	9.3% [4.1%-18%]	Uganda	1970	10-17yr child in one school (N=86)	NA	Throat	(120)
	0% [NA]				NA	Nasal	
4	1.5% [1.2%-2.0%]	UK	1974	Contacts (N=3,000)	57%	Throat and Nasal	(121)
5	14% [12%-16%]	US (Texas)	1969	Contacts (N=1,009)	NA	Throat	(115)
	9.8% [8.6%-11%]			All residents in the city (N=2,329)	NA	Throat	
6	4% [0.1%-22%]	Thailand	1996	HH contacts (N=23)	NA	Throat	(116)
	8% [3%-17%]			School contacts (N=74)	MA		
7	27% [25%-29%]	Indonesia	2012	Contacts <20yrs (N=1,739)	NA	Throat	(117)
8	3.1% [0%-7%]	Indonesia	2015	1-15 yr randomly sampled after the outbreak (N=279)	68%	Throat	(118)
					Toxigenic or non-toxigenic		
9	0% [NA]	Bulgaria, Finland, Greek, Ireland, Italy			-	Throat	(122)
	0.02% [0.0%-0.12%]	Estonia	2007 - 2008	Patients with an upper respiratory infection	Non-toxigenic	Throat	
	0.08% [0.1%-0.29%]	Latvia			Non-toxigenic	Throat	
	0.28% [0.11%-0.58%]	Latvia			Toxigenic	Throat	
	0.07% [0.01%-0.24%]	Lithuania			Non-toxigenic	Throat	
	0.14% [0.04%-0.34%]	Lithuania			Toxigenic	Throat	

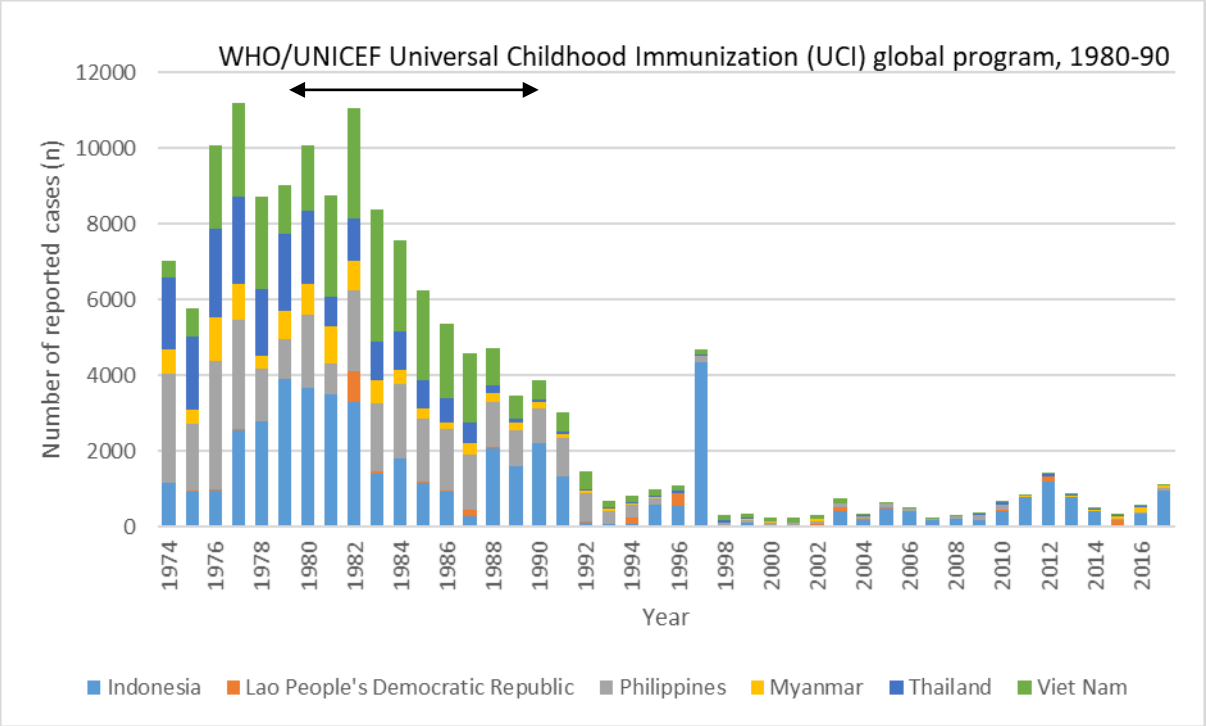
0.4% [0.2%-0.7%]	Turkey		Non-toxigenic	Throat
0.04% [0.01-0.1%]	UK		Non-toxigenic	Throat

NA: data not available

1.5. Diphtheria in Southeast Asia

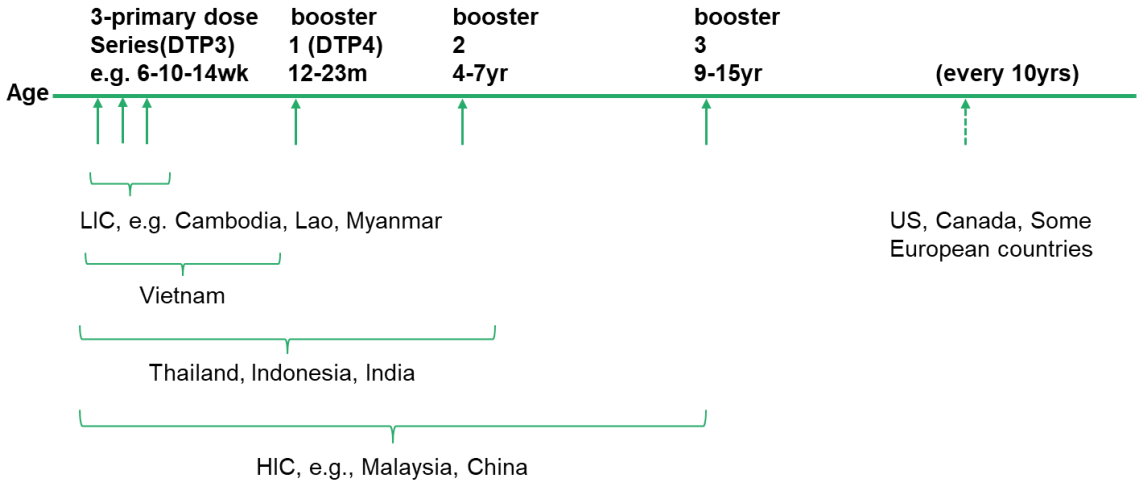
Since 2010, the global decline in the incidence of diphtheria cases has been reversed. There has been an increase in the number of reported cases in Southeast Asia (38). The highest numbers of diphtheria cases in Southeast Asia were reported in the Rohingya population (outbreak at the refugee camp of displaced people at the Myanmar-Bangladesh border) and Indonesia, followed by Lao PDR, Thailand, and the Philippines (123). Chapter 1 Figure 2 shows the trend of annual cases of diphtheria in Southeast Asia (123).

Chapter 1 Figure 2. Annual numbers of diphtheria cases were reported to WHO between 1974 and 2017 in the Southeast Asian region



The vaccination schedule and vaccine coverage in Southeast Asian countries vary. DTP schedules in different countries and the current WHO-recommended schedule (top row) are described in Chapter 1 Figure 3 (5, 38).

Chapter 1 Figure 3. DTP schedules in different countries and WHO recommended schedule



LIC: low-income countries HIC: high-income countries

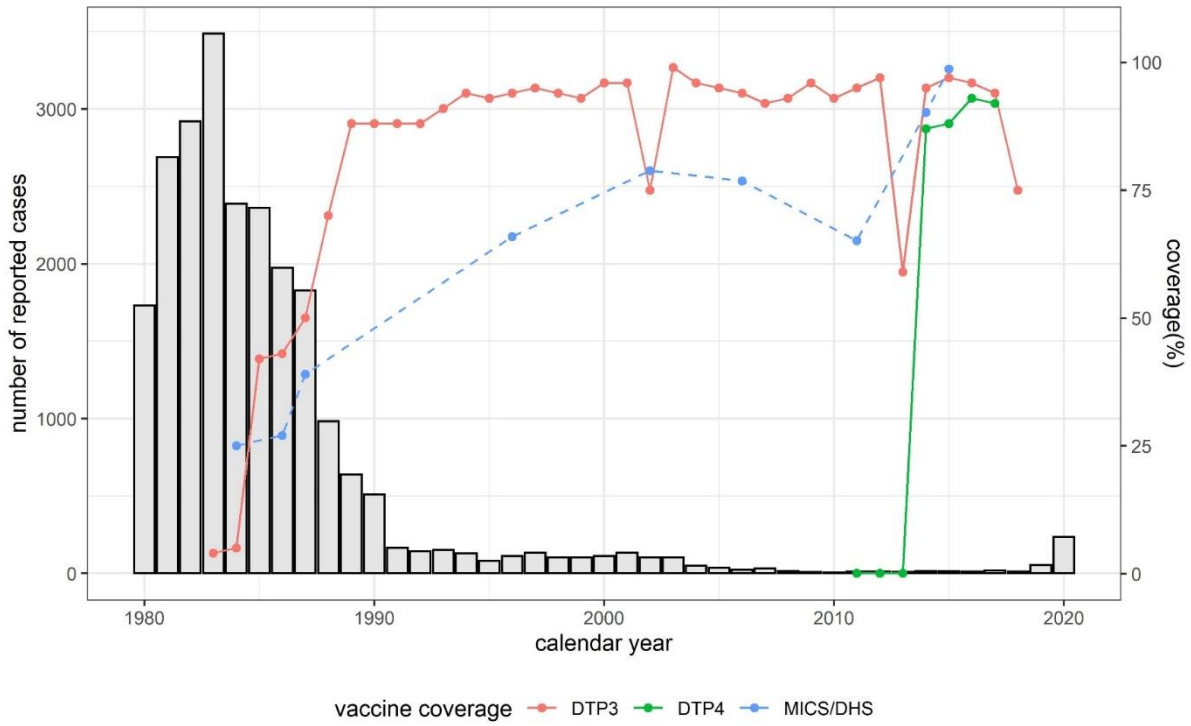
1.6. Diphtheria in Vietnam

In Vietnam, the DTP vaccine was introduced in 1981 and was replaced by the pentavalent vaccine in 2011. Three primary doses have been provided at 2, 3, and 4 months of age, and a booster dose at 18 months was introduced in 2012 (124). Similar to Western countries, the reported case numbers decreased sharply from 3,500 per year in 1983 to almost 0 in 2010 after the introduction of the DTP vaccine; however, clusters of cases have been reported since 2013 (123). There was a sudden drop in DTP3 coverage in 2002 due to low stock of the vaccine and in 2013 due to the suspension of DTP usage in the country after severe adverse events were reported (123). Since the introduction of Pentavalent vaccine in June 2010 till May 2013, 43 severe adverse events following immunisation (AEFI) were reported including 27 with a fatal outcome (125). According to the independent review of serious AEFI, none of them classified as having a consistent causal association with immunisation (125).

Administrative DTP3 coverage is continuously reported to be high in Vietnam; however, according to surveys (e.g., Demographic Health Survey [DHS] or Multiple Indicator Cluster Survey [MICS]), DTP3 coverage is not consistently high. National survey data suggest that DTP3 coverage gradually increased from 50% in the 1980s to 90% in the 2010s (126, 127). Furthermore, the surveys reported lower vaccination coverage in the central highland and Western regions in Vietnam, where most residents are from ethnic minority groups, though the precise local coverage in a specific area was difficult to obtain.

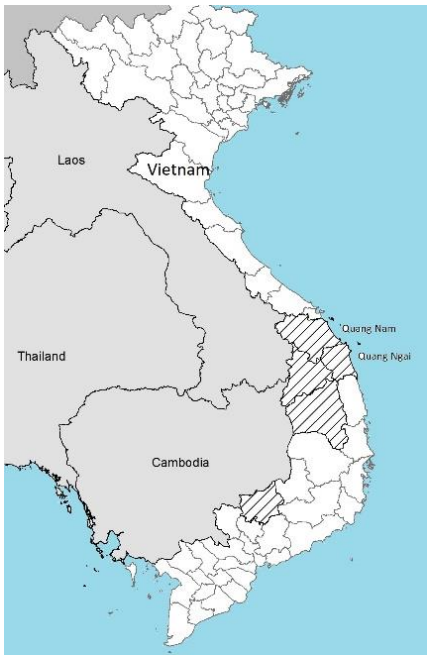
According to the WHO data, diphtheria incidence in Vietnam was reported at 11–21 annually between 2014 and 2019. The incidence rate of diphtheria in the Vietnamese population is 0.01–0.02 per 100,000 per year. In 2020, the annual diphtheria incidence exceeded 200 cases (Chapter 1 Figure 4). The cases were identified in rural areas where primary vaccine coverage is likely suboptimal. According to a report from the Pasteur Institute in Nha Trang and Tai-Nguyen Institute of Hygiene and Epidemiology, 116 cases were identified in Central and Western regions in Vietnam between 2014 and 2019 (Chapter 1 Figure 5). Of all the laboratory-confirmed cases since 2014, 13% were in children under 5 years old, 63% were in children 5–14 years old, and 24% were in individuals ≥ 15 years old.

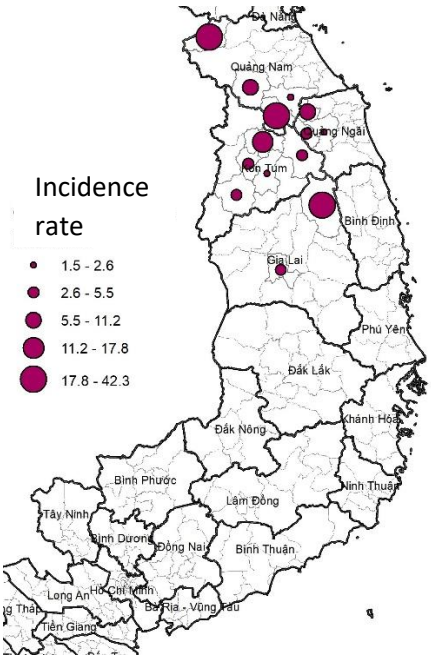
Chapter 1 Figure 4. The number of reported diphtheria cases and administrative and survey DTP3 coverage in Vietnam



(The figure was created based on the WHO database (5))

Chapter 1 Figure 5. Provinces where diphtheria cases were identified (left) and reported diphtheria incidence rate (per 100,000 population) by district between 2013-2018 (right) in Vietnam





2. Study aims

Diphtheria outbreaks in Southeast Asia and other parts of the world in the late 2010s raised the question of whether the outbreaks shared any epidemiological characteristics. Diphtheria vaccination for children in many LMICs is limited to three primary does, while the routine immunisation programmes in industrialised countries consist of five or six doses. The current population immunity in countries where three-dose primary series have been used for more than 30 years is unknown. Long or short-term suboptimal vaccination coverage and reduced natural immunity after the introduction of vaccination may have created susceptible populations. Although low vaccination coverage appears to contribute to current diphtheria outbreaks, it is crucial to clarify the immunity profile in the populations where diphtheria epidemics continue to occur. The carriage prevalence of the *Corynebacterium* species in LMICs, including carriers of non-toxigenic strains, who are mostly asymptomatic, has been largely under-reported.

Due to the repeated outbreaks, the Vietnamese Ministry of Health (MoH) has been discussing the introduction of a school-entry booster dose. This thesis aims to provide information and evidence for the discussion and decision-making regarding this booster.

The overall goal of my PhD research is to understand the epidemiology of diphtheria in Vietnam, to elucidate on the mechanism of diphtheria outbreaks over the last decade and to provide insight into future vaccination programmes in Vietnam and other countries.

The specific objectives are:

1) to describe the diphtheria outbreak in Vietnam between 2015 and 2018 (Chapter 3);
2) to measure diphtheria immunity in a population in which the vaccine uptake was consistently high with no school-entry booster dose (Chapters 5);
3) to estimate the optimal booster dose intervals for a routine immunisation programme (Chapters 4 and 5);
4) to measure the age-specific diphtheria carriage prevalence and seroprevalence in an epidemic-prone area (Chapter 6);
5) to identify the risk factors for bacterial carriage that potentially lead to diphtheria disease (Chapter 6); and
6) to validate the enzyme-linked immunosorbent assay (ELISA) assay as a method to detect anti-diphtheria toxoid IgG compared with a gold-standard method, the toxin neutralisation test (TNT), and to validate the seroprevalence obtained in Chapters 5 and 6 (Chapter 7).

3. Structure of the thesis

This thesis consists of eight chapters (Chapter 1 Table 3). Each chapter is described below.

Chapter 1 is an introduction to the thesis reviewing recent diphtheria outbreaks in the 2010s, including the reported cases and a schedule of diphtheria toxoid-containing vaccines in Vietnam and other countries.

Chapter 2 presents the results of literature reviews of background information on diphtheria, including microbiological features, natural history, treatment and outbreak response, transmission patterns (especially in the vaccinated population), and serology.

Chapter 3 describes the diphtheria outbreaks in rural provinces in Vietnam between 2015 and 2018. Age, sex, epidemiological links, laboratory confirmation, vaccination history of the cases, and multi-locus sequence type (MLST) of the isolates are reported. I describe the origin of the research questions and why this research series was planned.

Chapter 4 presents the results of a systematic review conducted to measure the waning rate of diphtheria immunity and the duration of protection after three, four, and five doses of DTP. In this chapter, the duration of protection after different numbers of DTP doses was estimated by analysing cross-sectional data collected in 15 European countries. The estimated duration of protection is useful for considering potential optimal intervals between the doses, as no such data are currently available.

Chapter 5 presents the results of a cross-sectional seroprevalence study of anti-diphtheria toxoid antibodies in a well-vaccinated community with a wide range of ages (0–55 years old), which had not been previously conducted in Vietnam. In this chapter, the duration of protection after receiving four doses of DTP is estimated from the longitudinal data of two cross-sectional surveys in Vietnam.

Chapter 6 describes the results of a cross-sectional carriage-prevalence and seroprevalence study. The carriage prevalence of *C. diphtheriae* and seroprevalence of anti-diphtheria toxoid antibodies in epidemic-prone areas in Vietnam is described. This chapter describes a significant difference in seroprotective levels by age in the population from the one described in Chapter 5.

Chapter 7 validates the ELISA anti-diphtheria antibody measurements assay by comparing them to results obtained via TNT, a gold-standard assay for detecting functional antibody levels in human serum samples, using the samples collected through the seroprevalence survey (Chapter 5). The seropositive and seronegative samples classified by ELISA in Chapter 5 are re-evaluated via TNT assay.

Chapter 8 discusses the overall results and provides recommendations for the vaccination programme in Vietnam.

Chapter 1 Table 3. Summary of each chapter in the thesis

Chapter	Objective	Method	Summary
1	Introduction to the research question	Literature review	Diphtheria is a global threat. Southeast Asian countries, including Vietnam, had several diphtheria outbreaks in the 2010s. There might be another reason for the concurrent outbreaks in addition to the low DTP3 coverage. This thesis aims to elucidate the mechanism of the recent diphtheria outbreak and to provide evidence for the vaccination strategy in Vietnam, including a school-entry booster dose.
2	Description of microbiological, serological, clinical and epidemiological features of diphtheria	Literature review	Classic diphtheria is a severe acute respiratory infectious disease transmitted person-to-person by droplets. Diphtheria toxin produced by toxigenic *C. diphtheriae* is the main pathogen of the disease in endemic areas, while *C. ulcerans* or non-toxigenic *C. diphtheriae* have become dominant in Western countries. The current DTP vaccine is one of the most effective vaccines to control bacterial disease in human history. However, the toxoid vaccine does not prevent infection transmission but instead prevents toxin-induced symptoms and death. The herd effect of the diphtheria toxoid vaccine exhibits a secondary effect of the vaccine that reduces the toxigenic strain in the upper respiratory tract of the host. The non-toxigenic strain can be converted to a toxigenic strain by lysogenic conversion of corynephage β carrying the *tox* gene, which is regulated by iron metabolism or other host factors. Eliminating corynephage β infection of *Corynebacterium* species reduces the incidence of diphtheria.

			There is no evidence that currently circulating strains in endemic areas escape from the vaccine at a molecular level. Therefore, the vaccine still plays a primary role in controlling diphtheria.
3	Description of the diphtheria outbreak in Vietnam	Cross-sectional study/ Retrospective cohort study	This study was conducted as a part of the routine EPI and national surveillance programme in Vietnam. Ninety-five suspected diphtheria cases reported from the Central region of Vietnam between 2015 and 2018 were investigated. Conclusions: • The 22 lab-confirmed cases were aged 3–27 years old; 73% were 5–14 years old. • Fully vaccinated cases (7 and 13 years) died, implying that immunity waned over time. • Cases were observed in areas with low DTP3 coverage. • Missing epidemiological links suggest that asymptomatic infection might have occurred during the study period. • Different MLSTs were identified in *C. diphtheriae* isolates at different locations and times, indicating ongoing multiple community transmission.
4	Quantification of waning diphtheria immunity and duration of protection after 3, 4, or 5 doses of DTP vaccine	Systematic review	Criteria of articles included in the systematic review: -Cross-sectional serosurvey data, stratified by single-year age group -Serology was measured by TNT or standardised by TNT. -No immunocompromised condition in the host -Targeted population in the routine national immunisation programme. Conclusions:

			• No data from LMICs met the criteria. • Serological data from 15 European countries were included for analyses. • The anti-diphtheria toxoid IgG level declined to the seroprotective threshold (0.1IU/ml) 2.5 years, 10.3 years, and 25.1 years after three, four, and five doses of DTP, respectively. • The results indicated potential optimal intervals of diphtheria toxoid-containing vaccine booster doses.
5	Seroprevalence and duration of protection after three or four doses of the DTP vaccine in a Vietnamese population with high vaccine uptake	Longitudinal (panel) study composed of two cross-sectional surveys	This study used pre-existing samples from an age-stratified seroprevalence survey conducted in a community with high DTP3 uptake in Vietnam in 2017. The study followed up the same participants at a 2-year interval. Conclusion: • The overall seroprevalence, defined as the proportion of individuals with anti-diphtheria toxoid IgG < 0.1 IU/ml in the population, was 26%. The lowest seroprevalence was 7% among children of school-age (6–15 years) children, which explains why the highest proportion of cases was found in this age group in Chapter 3. • The protection of the duration of vaccine-derived immunity was 4.3 years after the last DTP vaccination. Given that the last DTP dose was scheduled at 18 months, a booster dose at school-entry age (6 years in Vietnam), should be introduced to maintain protection against diphtheria.
6	Carriage prevalence and seroprevalence of diphtheria in	Cross-sectional study	The study was conducted in two rural districts where diphtheria cases have been identified, and where no supplemental immunisation activity (SIA) campaign has been implemented as of October 2019.

	an epidemic-prone area in Vietnam		Conclusion: • The seroprevalence was lowest in the 6–15 year age group (37%), which was similar to the 1–5-year age group (40%), probably due to low vaccine coverage, waning of immunity, and low seroconversion rate. • 1.4% of the study population was asymptomatic carriers of *C. diphtheriae.* Carriage prevalence was highest in the 1–5 year age group (4.5%), followed by the 617-year age group (2.5%). • 67% (18/27) of the isolated *C. diphtheriae* were non-toxigenic strains, suggesting that non-toxigenic diphtheria plays a role in transmission. • The low vaccine coverage produced low immunity among children 1–5 years old, and allowed them to be asymptomatic carriers. Children might have played a primary role in maintaining transmission. Unprotected school-age hosts became symptomatic as vaccine-derived immunity waned the most in this age group. This is probably the mechanism of the recent diphtheria outbreaks in Vietnam. • Improved DTP3 coverage and the introduction of a school-entry booster dose are recommended to stop transmission in Vietnam. • SIAs are recommended to target 1–17-year-old children and adolescents.
7	Validating the immunity measured by ELISA via neutralisation assay	Validation study (parallel comparison) Misclassification bias correction	This study compared diphtheria toxoid antibody (IgG) levels measured in serum and dried blood samples by ELISA (IBL) assay to the IgG levels measured in the serum by TNT, a gold-standard method for detecting functional antibodies in the human serum. Conclusions:

			• Dried blood spot (DBS) was confirmed as a field-friendly alternative tool for diphtheria seroepidemiological study. • An ELISA cut-off value of 0.1IU/ml accurately classified the protected individuals and estimated the protection level in the population. • Seroprevalence based on the ELISA cut-off value of 0.01IU/ml overestimated the protection level in the population. • One-third of the population in a well-vaccinated community in Vietnam was susceptible after adjusting the protection level by using TNT.
8	Discussion and conclusion		• Only 26% (estimated 20% based on the comparison with TNT measurements) of the population had long-term protection against diphtheria in a well-vaccinated community in Vietnam. Individuals aged 6–15 years were especially susceptible, which corresponds to the age of diphtheria cases in the diphtheria epidemic area of Vietnam. • The average seroprevalence was 68% among children aged 0–5 years, although the reported routine DTP3 coverages in the last 5 years was over 90%, except in 2013. The relatively low seroprevalence was due to the rapid waning of anti-diphtheria toxoid antibody levels. DTP-derived anti-diphtheria toxoid antibodies declined quickly to 0.1 IU/ml within 5 years after the fourth dose, which was given at 18 months of age. • The seroprevalence among children aged 1–5 years in the diphtheria epidemic-prone area was 40%, which was significantly lower than the 68% estimated in the well-vaccinated area. The seroprevalence in individuals aged above 5 years was higher in the epidemic-prone area compared with

			the well-vaccinated area. This observation suggests that low seroprevalence, and thus low vaccination coverage, among young children is key for diphtheria outbreaks. • The different seroprotection levels in non-epidemic and epidemic-prone areas suggest that low protection among children allows them to become carriers and thereby, continue transmission. • A school-entry booster dose, specifically at age 6 years, is recommended to maintain the protection in the community against diphtheria in Vietnam. At the same time, DTP3 coverage must be maintained high in the entire country.

Chapter 2: Literature review

Chapter 2 summarises the biological, microbiological, clinical, and epidemiological features of diphtheria to understand the pathogenicity of *Corynebacterium* species, transmission patterns, a different forms of diphtheria, preventive method, and treatment to aid in discussions regarding diphtheria control strategies.

1. Classification of *Corynebacterium* species

C. diphtheriae is a club-shaped Gram-positive bacillus and is usually the causative agent of diphtheria (112). Three *Corynebacterium species* infect humans. Diphtheria toxin produced by toxigenic *Corynebacterium species* is pathogenic for many animals; however, humans are the only host for *C. diphtheriae*. In contrast, *C. ulcerans* and *C. pseudotuberculosis* are zoonotic pathogens that infect both humans and animals. *C. ulcerans* and *C. pseudotuberculosis* typically cause ulcerative lesions in cattle and horses. Human infections with these species are rare and are traditionally reported among rural populations that have direct contact with domestic livestock animals or who consumed unpasteurised dairy products (103). Recent reports showed that *C. diphtheriae* might also have been transmitted between humans and domestic or wild animals (128). In addition to *C. diphtheria*, *C. ulcerans* and *C. pseudotuberculosis* can be converted to toxigenicity and produce diphtheria toxin. This finding is important because it indicates that non-*C. diphtheriae* strains may potentially cause outbreaks in humans.

Toxigenic strains that have *tox* genes and produce toxins and non-toxigenic strains that do not produce toxins have been well distinguished in *Corynebacterium* infections. However, a NTTB strain identified in the 1990s was found to be pathogenic to human hosts. NTTB strains contain *tox* genes; however, mutation in the A-subunit of the gene prevents *tox* gene expression (128). NTTB strains invade human cells directly but do not cause disease through a bacteria-produced exotoxin. Recently, NTTB strains have been increasingly recognised across Europe (128).

Subtyping of *C. diphtheriae* and other *Corynebacterium* species is helpful for epidemiological surveillance, but its practical use is limited due to the labour-intensive laboratory work and low discriminatory power and reproducibility (101). Traditionally, serotyping, phage typing, and bacteriocin typing have been used to differentiate *C. diphtheriae* (129). However, phage typing or bacteriocin typing was no longer valuable for tracing transmission (130). There are four major biovars (previously called serotypes) of *C. diphtheriae* that are classified based on the biochemical characteristics: *mitis, gravis, intermedius*, and *belfanti* (130). Originally, it was thought that *gravis* caused more severe disease than *intermedius,* and *intermedius*

caused more severe disease than *mitis*. It was later determined that there was no correlation between disease severity and biovars or between biovars and genomic characteristics (131).

Ribotyping, which is allocated a geographical name based on the location of isolation, used to be a gold standard for genotyping (108). Since the 1980s, several different molecular methods, such as Southern hybridisation, and multi-locus enzyme electrophoresis (MEE), have evolved (132, 133). More recently, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF) (134, 135), MLST (136), and whole-genome sequencing (WGS) have used to identify the genetic characteristics and have replaced ribotyping.

MLST is appropriate for investigating long-term evolutionary dynamics and transmission paths, and sequence types (STs) are consistent with the ribotypes despite STs not correlating with the biovars or disease severity (136). Currently, there are 384 reference STs available on the MLST website (http://pubmlst.org/cdiphtheriae/) (108). WGS of *C. diphtheriae* was completed for the first time in 2003. WGS analyses revealed that the diverse phylogeographical structure of *C. diphtheriae* correlates with area-specific endemic variants whose circulation is strongly influenced by vaccination (137).

2. Pathogenicity of *Corynebacterium*

2.1. Corynephage and lysogenic conversion of *Corynebacterium*

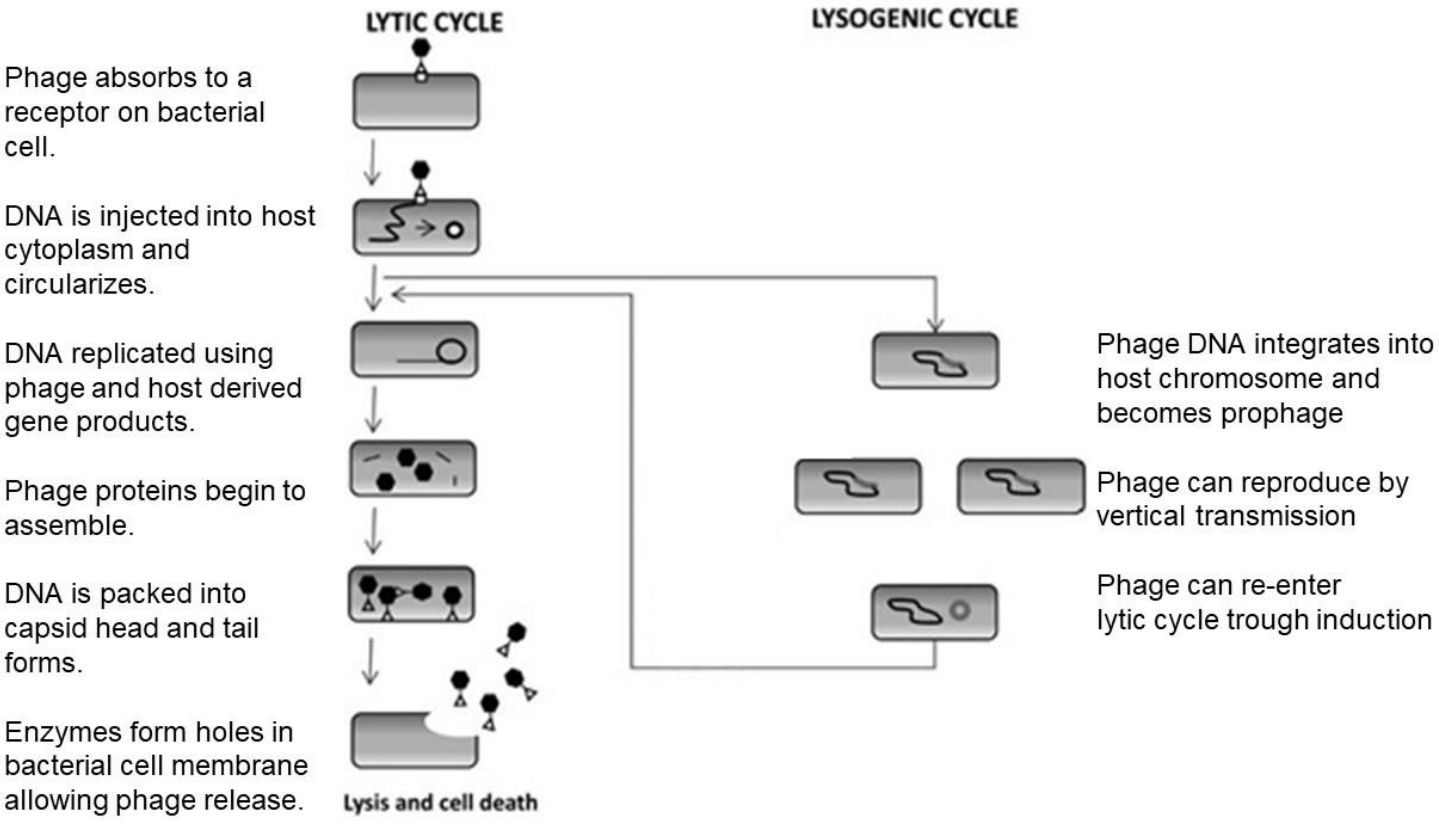
C. diphtheriae often grows in the nasopharynx and creates a fibrinous membrane overlying a painful, haemorrhagic, and necrotic lesion. This typical pathogenic lesion created by the exotoxin leads to the symptoms of classical respiratory diphtheria. Other clinical manifestations of diphtheria, such as myocarditis and peripheral nerve paralysis, are also caused by the exotoxin, which circulates inside the body and damages the cells in organs. There is no specific target organ for diphtheria toxin (138).

Freeman discovered that non-toxigenic *C. diphtheriae* was converted to a toxigenic form via infection by a bacteriophage, corynephage β (139). Later, Uchida et al. found that the toxin was coded by a corynephage gene (140). Bacteriophages are viruses that infect bacteria. There are an estimated 10^{31} bacteriophages on the planet, and they are the most abundant form of life (141). Furthermore, they are ubiquitous and have been found in every environment, including the sea and freshwater, tropical and desert soils, hot springs, sewage, human intestines, and the oral cavity (142). Some phage genes are known to increase the survival of host bacteria (143), such as corynephage β for the survival of *C. diphtheriae*. Corynephage β plays a crucial role in the infection and transmission of *C. diphtheriae*.

Temperate (lysogenic) phages integrate their genome into the host chromosome and become a prophages. Lysogenic infection by a bacteriophage and subsequent expression of phage-encoded genes by the host is called lysogenic conversion. Lysogenised *Corynebacterium* species with a prophage encoding the *tox* gene produce diphtheria toxin. Therefore, elimination of the toxin-coding prophage from the bacteria results in loss of the ability to produce diphtheria toxin (144).

In contrast to lysogenic infection, virulent (lytic) phages take control of host bacteria, and the lytic enzyme causes cell lysis to release lytic phage progeny. The phage DNA replicates along with the host cell (lysogen) as a prophage and is maintained in the bacterial population (141). A schema of the lysogenic and lytic cycles of a bacteriophage is shown in Chapter 2 Figure 1.

Chapter 2 Figure 1. A lysogenic cycle and a lytic cycle of bacteriophage.
(adapted from Davies et al. 2016 (141))



A lysogenic phage can switch to a lytic life cycle (141). The balance between lytic and lysogenic states largely depends on the metabolic condition of the host cell. In starving cells, lysogenic phages tend to stay in a lysogenic state to survive during resource limited periods.

The switch from the lysogenic cycle to the lytic cycle occurs spontaneously and is often triggered by DNA damage to the host cells, which is often caused by ultraviolet (UV) light, reactive oxygen compounds, and several antibiotics that target DNA replication—this is thought to be a survival strategy of phages. Phages escape from a host cell at risk of death by switching to the lytic cycle.

Recent evidence suggests that the phage-encoded exotoxin genes may be maintained in the environment either as a free phage or in alternative bacterial hosts (142). Phages can survive outside of their microbial hosts for extended periods, often in harsh physical and chemical environments. Therefore, diphtheria toxin genes (*dtx*) may be exchanged and maintained in *Corynebacterium* and alternative bacterial hosts.

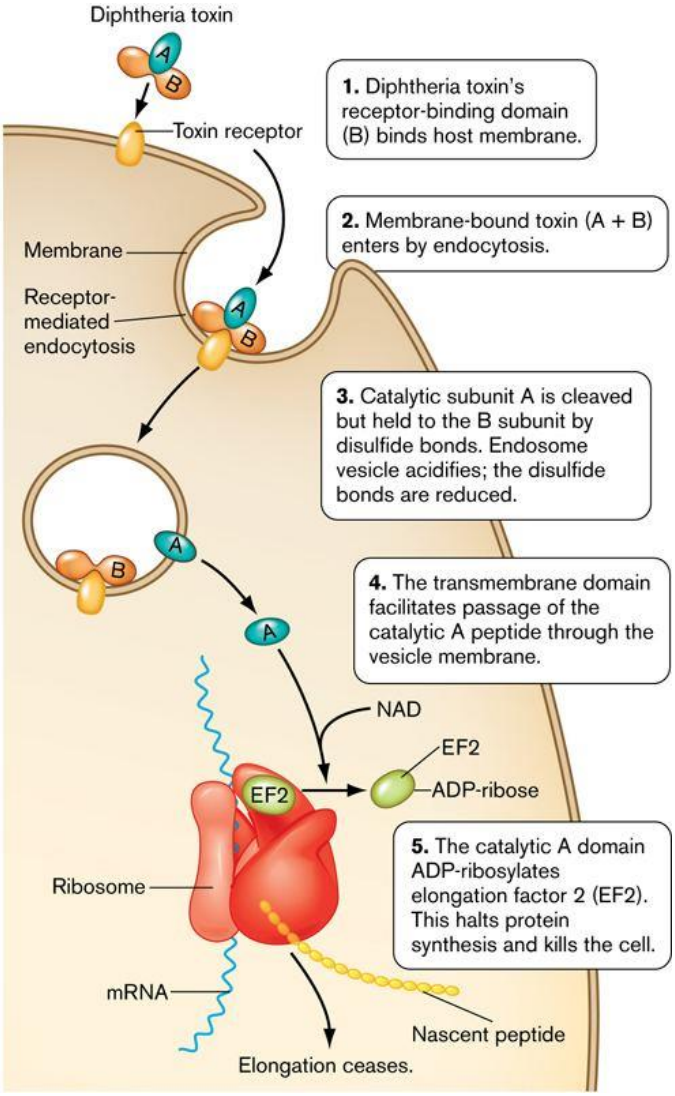
Pappenheimer found that the *dtx* gene is exchanged between non-toxigenic strains and toxigenic strains in the upper respiratory tract in human hosts (138). It is also possible that reservoirs of phage-encoded exotoxin genes are maintained in the upper respiratory tract in human hosts, and transduction of a phage carrying *dtx* into a bacterium as part of normal bacterial flora in the upper respiratory tract will lead to disease (142).

2.2. Regulation of diphtheria toxin production

Diphtheria toxin is the primary virulence factor for *Corynebacterium*. Mature extracellular diphtheria toxin produced by *C. diphtheriae* is a ~58kDa polypeptide that has two internal disulphide bonds (144) to which an amino-terminal fragment A and a carboxyl-terminal fragment B are connected. These two fragments are also called diphtheria toxin A subunit and B subunit. The toxin is endocytosed and processed within the cell, and the internal bonds are reduced by acidic endosome vesicles. A subunit introduced into the cytoplasm acts catalytically to transfer an adenosine diphosphate ribose (ADPR) moiety from nicotinamide adenine dinucleotide (NAD) to elongation factor 2 (EF-2) (145). Ribosylated EF-2 inhibits protein synthesis and leads to cell death (146). The mechanism by which diphtheria toxin damages the cells and organs in the host is described in Chapter 2 Figure 2.

Chapter 2 Figure 2. Mode of action of diphtheria toxin

(adapted from https://alchetron.com/Diphtheria-toxin)



Pearsons and Groman described how toxigenic strains could emerge after exposure to a phage originating from non-toxigenic strains (147, 148). They suggested that non-toxigenic strains might carry all or part of the *tox* gene, and under certain conditions, a fully expressed gene could be recovered. Toxigenic strains of *C. diphtheriae* expressing the *tox* gene produce diphtheria toxin. Toxin production by toxigenic strains is regulated by an iron-dependent regulatory protein called diphtheria toxin repressor protein (DtxR) (149). DtxR regulates not only the expression of diphtheria toxin but also siderophore, which scavenges iron from a host for bacteria to survive (150). Because DtxR utilises iron as a co-repressor to inhibit transcription of the *tox* gene, the production of diphtheria toxin is decreased under high-iron conditions (144, 149).

DtxR suppresses the production of toxins. In contrast, deficiency of the *dtxR* gene reduces the expression of DtxR, resulting in increased production of toxin. Groman et al. assumed

that non-toxigenic strains possessed functional *dtxR* genes, as it explained why some non-toxigenic strains carrying *tox* genes did not produce the toxin (148). De Zoysa et al. confirmed that all non-toxigenic *C. diphtheriae* strains circulating in the UK in the 1990s carried the *dtxR* gene (151). Furthermore, it was demonstrated that non-toxigenic strains become a potential reservoir for the *tox* gene (152). Therefore, there is a risk of re-emergence of toxigenic strains in areas where non-toxigenic strains are circulating.

3. Clinical manifestation of diphtheria

Diphtheria is an acute bacterial infectious disease caused by *Corynebacterium* species, mainly *C. diphtheriae*. The toxin secreted by *C. diphtheriae* induces upper respiratory stenosis or myocarditis, which can have a mortality rate of 5–20% without treatment (153).

3.1. Symptoms

One severe and typical form of diphtheria is pharyngeal diphtheria. Common symptoms of pharyngeal diphtheria are sore throat, fever, and difficulty breathing and swallowing. After infection, a membrane (pseudo-membrane) forms across the tonsils and spreads to the pharynx. The patient may die 7-10 days after the onset of symptoms as the membrane obstructs the airway or the diphtheria toxins spread through the body, (112).

The most common complication of diphtheria is myocarditis, which occurs between 14 to 21 days after the onset of symptoms and generally has a poor prognosis. The complication rate of myocarditis is the highest among individuals 5–19 years old (8.5%) and adults (6.5%) and is the lowest among children younger than 5 years old (3.5%) (11). The second most common complication, paralysis involving the peripheral nerves, eye muscle, soft palate, or diaphragm, is typically found in 12.4% of cases (154). Paralysis will completely resolve unless the central nervous system or respiratory muscles are involved (112).

The incubation period, symptomatic period, infectious period, latent period, and natural history of diphtheria infection are summarised in Chapter 2 Table 1.

Chapter 2 Table 1. The incubation period, symptomatic period, infectious period, asymptomatic carrier status, Latent period, serial interval, recurrent infection rate, and CFR in the pre-vaccination period

	Value	Ref
Incubation period	1-6 days 1.7 days (95% CI, 1.0-3.0)	(Plotkin 2018) (155) (Truelove 2019) (156)
Symptomatic period	5.6 days (95%CI: 3.9-7.4 days)	(personal communication: Dr. Nobuo Saito, Oita University)
Bacterial carriage duration	14 days (50%), 28 days (75%), 1-2 months (1-8%)	(Plotkin 2018) (155)

=infectious period	17 days (95% CI, 16-18)	(Truelove 2019) (156)
Asymptomatic carriage duration	18 days (95% CI, 13-25)	(Truelove 2019) (156)
Proportion of Asymptomatic carrier	93% (post-vaccination) 97% (post-vaccination) 30% (pre-vaccination)	(Ukraine 2002-2009) (Miller 1970, US) (157) (Truelove 2019) (156)
Infectiousness of asymptomatic carrier	20% (< 5 years) to 5% (> 20 years) of symptomatic infection 24 % of symptomatic infection	(Doull 1925, UK) (158) (Truelove 2019) (156)
Latent period	2-5 days	(CDC 2015) (153)
Serial interval	8.3 days (95%CI 7.65 - 9.05 days)	(Stocks 1930) (159)
Time till death:		
Early onset shock	10 days +	(Christie 1985)(112)
Pharyngeal diphtheria	7-10 days	(Christie 1985)(112)
Myocarditis	14-21 days +	(Christie 1985)(112)
Non-specific overall	5 days (95%CI: 4.0-6.4 days)	(personal communication: Dr. Nobuo Saito, Oita University)
Recurrent infection:	Rate=0.00385 per year Rate=0.000652 per year	(Crum 1917) (154) (Crum 1917) (154)
CFR:	8% in all age groups 5-20 %	(Crum 1917) (154) (CDC 2015) (153)

3.2. Incidence and mortality rate

Diphtheria incidence varies by age. Before the widespread introduction of vaccination, children, especially 1–4 years old, were the most susceptible (155). Records from the USA in the early 20th century, shortly after child education in the primary-school was legalised, showed that 5–9-year-old children had the highest incidence of diphtheria, then the incidence decreased after 10 years of age (154).

The CFR also varies by age. Deacon calculated age-specific CFR based on the 31,208 cases and 2,458 deaths reported in Michigan from 1910 to 1914 (160). The CFR decreases exponentially with age: highest at 0–1 years (62%), 2–3 years (21%), 5–9 years (10%), 10 years (5%), 20 years (2%), and lowest at 30 years (1%) (154). Overall CFR was reported to be 8% (160). The mortality rate due to diphtheria among 3–10-year-old children increased after elementary education became mandatory in the USA in the 19th century. Increased frequency and intensity of contact in a school appeared to be a factor that influenced the increased incidence and mortality observed among school-age children (161).

Different incidence and mortality rates of diphtheria by sex have also been reported (154). The difference in incidence is not marked, but the mortality rate in adult women (1.5 per

100,000) is 1.5-fold higher than in adult men (1.1 per 100,000). Crum suggested that this difference might be due to the social roles undertaken by women in caring for sick people (154). Nevertheless, a difference in biological immunity between sex may exist as high susceptibility in women was reported even after the introduction of the DTP vaccine (162-165).

3.3. Case management and outbreak response

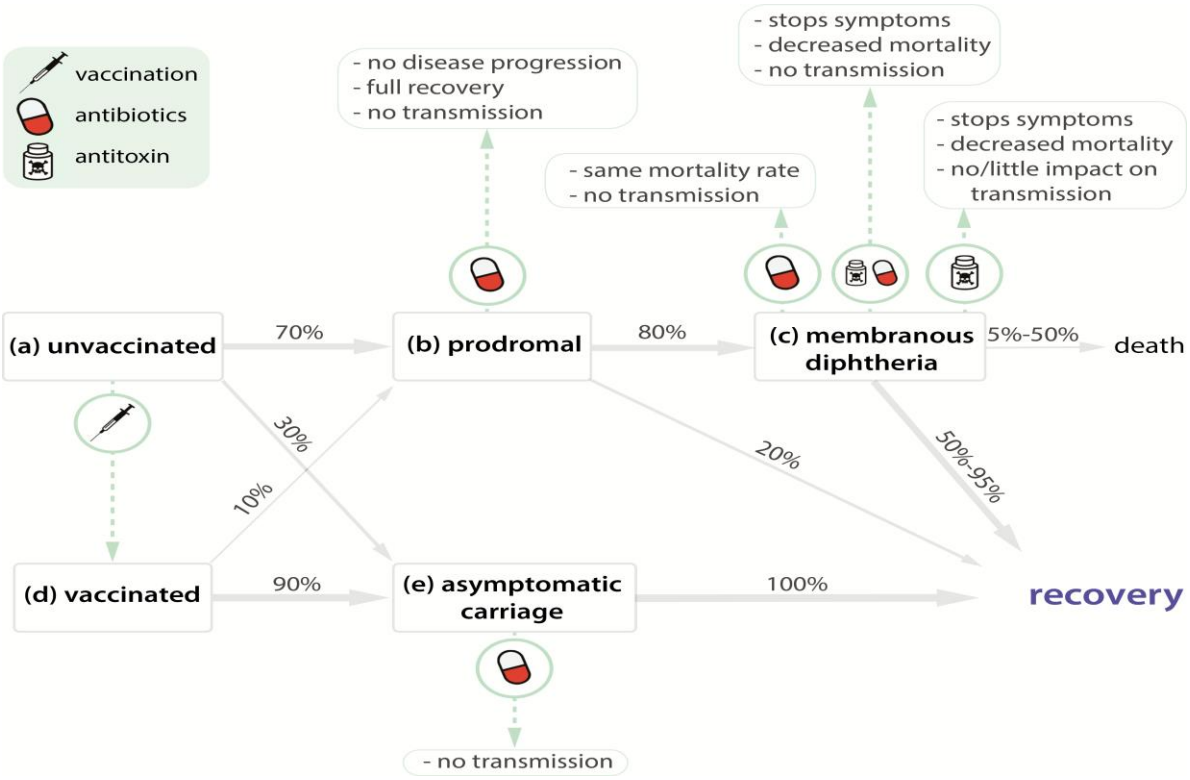
Diphtheria antitoxin (DAT) is an effective treatment for diphtheria, and early initiation of treatment is critical to increasing survival (154). Although DAT is highly effective in preventing death, it is currently unavailable in many parts of the world (38). A monoclonal antibody is currently under development as an alternative treatment to DAT (166).

Antibiotics do not mitigate symptoms if administered after bacteria colonise the host and produce toxins. Asymptomatic carriers can be treated by oral erythromycin or penicillin. Usually, 1 week of treatment is sufficient for carriers to prevent transmission. It was reported that secondary transmission could be prevented if three-quarters of contacts of cases were treated with antibiotics (167). Antibiotic resistance to diphtheria was once uncommon (146); however, resistance to penicillin and/or erythromycin has been reported in regions around the world in the last 10 years (12, 75, 168-171).

SIAs using the diphtheria toxoid vaccine have been conducted during outbreaks in many countries, including Indonesia, Haiti, Vietnam, and Yemen (19, 26, 53, 55). The WHO surveillance guidelines suggest including adults in target SIAs; however, the target age is not specified (172). Recent SIAs have targeted children aged 1–15 years in Haiti, 1–19 years in Indonesia, and 1–40 years in Vietnam(19, 26, 53).

It should be noted that the toxoid vaccine will not prevent infection in addition to carrier status with *Corynebacterium* species; therefore, a vaccine is not effective in stopping transmission immediately during an outbreak. In contrast, antibiotic administration reduces the carriage of bacteria in the host and secondary transmission from the host. Therefore, administration of prophylactic antibiotics for contacts is recommended to control diphtheria outbreaks. Chapter 2 Figure 3 shows the effective treatment and prevention strategies for diphtheria (156).

Chapter 2 Figure 3. Treatment and preventive strategy in the different stages of infection in individuals



The toxoid vaccine prevents the development of toxin-induced symptoms and death; however, it does not prevent the disease caused by non-toxigenic strains. Future vaccine development with universal surface proteins that may be more effective in reducing carriage and the invasive disease caused by non-toxigenic strains could potentially help control diphtheria (108). Another problem with the current DTP vaccine is a severe local reaction after multiple booster doses. Cross-reacting material (CRM) was thought to be a candidate for future vaccines as it will be less reactogenic when used as a booster dose (130).

4. Transmission of *Corynebacterium* species

4.1. Mode of transmission

The mode of transmission of diphtheria is by droplets. Diphtheria infection spreads person-to-person by intimate respiratory or physical contact, such as between family members living in the same household or children living in dormitories (155, 173, 174). Belsey suggested that skin carriers may play a greater role in continuous transmission than respiratory carriers in subtropical areas (175); however, the prevalence of skin carriers is unknown. Fomite transmission in respiratory diphtheria was suspected to occur in impoverished areas in the USA but has not yet been proven (175).

4.2. Cutaneous diphtheria as a source of continuous transmission

Cutaneous diphtheria is another form of *Corynebacterium* infection. Deep, sharply demarcated, long-lasting, punched-out ulcers are caused by *Corynebacterium* infection. Cutaneous diphtheria was discovered after the extensive outbreak of respiratory diphtheria in Europe (176). Approximately 1% of nasopharyngeal diphtheria was once associated with the complication of cutaneous diphtheria in temperate zones (177). In contrast, skin lesions in tropical areas occur more frequently in subjects with respiratory diphtheria (177). Both toxigenic and non-toxigenic strains of *C. diphtheriae* have been identified in infected cutaneous lesions (178).

The prevalence of cutaneous diphtheria has varied by geographical location and time. Several historical studies reported the proportion of diphtheria infection among skin lesions, which is summarised in Chapter 2 Table 2.

Chapter 2 Table 2. The proportion of diphtheria infection among individuals with skin lesions who visited outpatient-clinics or in population

Proportion of diphtheria infection among individuals with skin lesions		country	year	Population (N)	non-toxigenic proportion among isolates	Ref
1	5-11%	Colombia	1968	black and mestizo children	64% (41-91%)	(179)
2	32.0% [27-37%]	Indonesia	1965	Asian (N=394)	98.50%	
3	31.8% [25-39%]	Cook Islands	1959	Maori (N=170)		
4	40.8% [31-51%]	Ceylon	1968	Asian (N=98)		(180)
5	28.7% [24-34%]	Samoa	1955	Samoan(N=278)		
6	21.5%[19-24%]	Pacific island	1945	White soldiers (N=805)	16%	(181)
7	67.5%[60-74%]	Middle East	1919	White soldiers (N=191)		
8	32.5%[29-37%]	USSR	1956	Whites (N=889)		
9	13.6%[7.6-16%]	USA (LA, AL)	1969	White & Black (N=268)	34%	(175)
10	68%[57-78%]	USA (TX)	1947	Soldiers (N=82)		(182)
11	33%[10-65%]	USA (TX)	1969	contacts during outbreak (N=12)		(115)
12	50% [41-58%]	Uganda	1970	0-18yr with skin lesion	96%	(120)
13	64%[59.5-68%]	Myanmar	1979	0-19 yr patients in dermatology clinic (N=493)		(183)
	63%[52-74%]			< 1yr (N=80)		
	64%[59-69%]			1-9 yr (N=371)		
	71%[52-87%]			10-12 yr (N=31)		

	27%[6-61%]			12-19 yr (N=11)	
14	5.5% [1.8-12.4%]	Tanzania	1973	person with skin lesion randomly selected 0-19 yr (N=96)	(184)
	0%[NA]			< 1yr (N=24)	(184)
	5%[0.6-17%]			1-5 yr (N=40)	
	21%[5-50%]			5-12yr (N=14)	

Populations in tropical areas were thought to act as reservoirs of the bacilli (178). It was recognised that acute skin infection or colonisation with *Corynebacterium* occurs in both intact and pre-existing skin lesions accompanied by nasopharyngeal diphtheria. However, chronic skin infection always occurs superimposed on a pre-existing skin lesion (178). Protein or vitamin B2 complex deficiency (riboflavin insufficiency) co-existing with respiratory diphtheria was suspected to be a potential risk factor for cutaneous diphtheria; however, this has not been proven (176). The risk of skin infection appears to increase with low socioeconomic status and poor living conditions (120). Cutaneous carriage of *C. diphtheriae* can act as a silent reservoir for the organism. It has been reported that person-to-person transmission from infected skin sites was more effective than respiratory tract transmission in causing respiratory diphtheria (185).

An investigation of cutaneous diphtheria in a war camp in Myanmar in the 1940s suggested that some pre-conditions were necessary for an epidemic of cutaneous diphtheria (176):

- A certain proportion of the population remained susceptible.
- A hot and humid climate, in which moist skin and activity of the sweat glands provide a favourable environment for bacilli to enter, or a desert climate.
- A source of infection or existence of a reservoir of respiratory or cutaneous diphtheria.
- Lack of personal hygiene, close contact, and pre-existing skin lesions, which could be in any form (e.g., abrasions, cuts, bites, stings, scabies, ulcerative dermatitis, burns, or wounds).

These conditions were also identified as risk factors for the outbreak of respiratory diphtheria, which is discussed in Chapter 1.

4.3. Transmission of *C. diphtheriae* after introduction of the vaccine

Diphtheria was one of the leading causes of child death in Europe until the early 20th century. In 1890, Behring and Kitasato reported that toxins treated with iodine trichloride successfully immunised animals (186). In 1924, a formol toxoid was developed by Ramon as a toxoid vaccine for humans, although widespread use of toxoid vaccines would not be established until the late 1930s (1). The incidence of diphtheria began to decline before the

vaccination programme started, probably due to improved living standards and personal hygiene in less crowded households (187). After the toxoid vaccine was introduced, the disease nearly disappeared (38).

Diphtheria is probably the only bacterial infectious disease that has almost been eradicated by active immunisation. The vaccine reduced the incidence rate from 168.0 to 24.5 per 100,000 population and the mortality rate from 6.4% to 0.9% during outbreaks in Canada in the 1940s. The prevalence of symptomatic infection was 3.5-fold lower, and the mortality rate was 25-fold lower in immunised individuals than in unimmunised individuals in the UK in 1943 (188). Vaccine effectiveness was estimated to be 97% (95% CI:86–99) among children aged 0–2 years, 96% (95% CI:87–99) among children aged 3–5 years, and 90% (95% CI:73–96) among children aged 6–14 years who received three doses of DTP (39).

The protective immunity induced by the toxoid vaccine is effective against the toxin protein that is responsible for the pathogenesis of diphtheria. All toxigenic strains of *C. diphtheriae* produce immunologically identical toxins; therefore, strain-specific vaccines are not necessary (130). The diphtheria toxoid vaccine effectively prevents disease caused by all strains (189). The investigation of multiple strains identified in the former Soviet Union confirmed that diphtheria toxin is highly conserved at the amino acid level, while some heterogeneity was found at the DNA level (190). The study reaffirmed that the current toxoid vaccine should protect individuals from all toxin producing strains.

Changes in diphtheria incidence in Romania after mass vaccination demonstrated the effect of the toxoid vaccine (129). Between 1958 and 1972, 30 million doses of toxoid vaccine were administered; the proportion of protected population increased from 60% to 97% in the same period. At the same time, the incidence rate of diphtheria dropped from 600 to 1 in the population of 10 million. In 1958, 90% of *C. diphtheriae* isolated from humans were toxigenic; in 1972, more than 95% of isolates were non-toxigenic. Toxigenic strains started disappearing several years after the decrease in disease incidence, until finally, *tox* genes were virtually eliminated from human carriers (130). It should be noted that while the carrier rate did not decrease, the corynephages carrying the *tox* gene disappeared in Romania (130).

Toxigenic and non-toxigenic strains of *C. diphtheriae* within the same host are genetically identical (132). In a highly susceptible population, toxigenic strains will have a strong selective advantage compared with non-toxigenic strains. Diphtheria toxin alters local tissues, promoting colonisation and reproduction of bacteria, thereby, contributing to ease of transmission. If toxigenic strains were introduced into an immunised population, they would have no advantage over the normal bacterial flora and would likely fail to colonise or transmit

(191). This explains the decrease in the circulation of toxigenic *C. diphtheriae* strains in highly immunised populations.

Regardless of the immunisation status of an individual, if toxigenic strains reach the upper respiratory tract of an individual who already carries non-toxigenic *C. diphtheriae*, lysogenic conversion may occur within the upper respiratory tract. Then, the *tox* gene carrying corynephages may spread within its new bacterial host and further spread to other hosts carrying non-toxigenic strains in the community. This appears to be a mechanism of how *C. diphtheriae* spreads in a vaccinated community (138).

On an individual basis, the effectiveness of the diphtheria toxoid vaccine against the disease is incomplete; vaccinated individuals have a more mild disease when infected compared with non-vaccinated individuals (157, 192, 193). Faulted vaccines are unlikely to be the reason for incomplete protection. The combination of incomplete protection in fully vaccinated individuals and no protection in incompletely vaccinated individuals maintains susceptibility to diphtheria in communities. In these communities, the infection spreads when the organisms are re-introduced (43, 115, 194-196).

The necessity of booster vaccination was already being discussed in the 1940s in Europe (197). As a result, multiple booster doses were consecutively introduced in industrialised countries. Only the countries that had achieved high vaccination coverage in all age groups eliminated diphtheria by reducing the prevalence of *tox* gene-bearing *C. diphtheriae*. The herd effect or protection of nonimmune individuals of the diphtheria toxoid vaccine is due to the reduced transmission of *tox* gene-bearing *C. diphtheriae* (194).

5. Serology of diphtheria antitoxin

Acquired immunity to diphtheria was measured by the Schick test before modern laboratory methods were developed. The procedure of the Schick test is as follows: a small amount of diluted diphtheria toxin is injected into one arm, and the heat-inactivated toxin is injected into the other arm as a control. The Schick test is positive if the red reaction is observed only in the tested arm, which indicates that the person is susceptible.

A neutralisation assay using Vero cells was developed as an alternative to the Schick test; more recently, ELISA became available as a low-cost and minimally labour-intensive method. The TNT assay is the gold-standard for detecting antibodies neutralising the diphtheria toxin. ELISA is less reliable for quantifying the anti-diphtheria toxoid antibody levels than TNT, especially when antibody levels are low. However, the use of ELISA is reasonable for population-level seroepidemiological studies considering its advantages, such as shorter processing time and less work.

Antibody titres measured via in vitro assay do not completely agree with the Schick test; however, IgG levels ≥ 0.01 IU/ml measured via TNT are considered equivalent to a negative Schick test. As a current consensus, individuals with antibody levels < 0.01IU/ml are susceptible, ≥0.01IU/ml have basic (some degree of) protection, and ≥0.1IU/ml have long-term (full) protection (172).

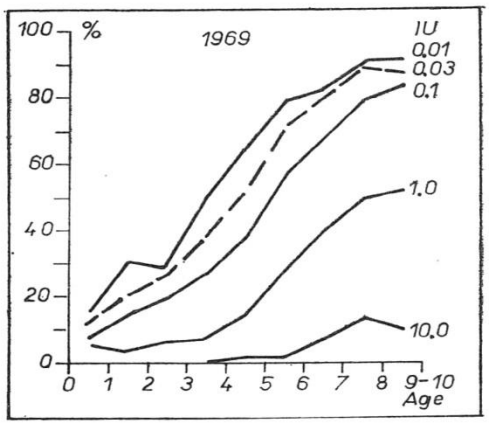
Before DTP vaccines were widely available, host immunity was acquired and maintained by repeated natural infections or exposures (154). Young children were the most susceptible, and adults were protected. After the vaccine was introduced, the immunity levels by different ages and the age of cases shifted depending on the extent of vaccination coverage, (Chapter 1). As vaccine-induced immunity wanes after vaccination of with DTP (198), the infection risk increases over time since the last vaccination (193). The individuals who received three doses of DTP were thought to be protected for 10 years after the last vaccination (199).

The seropositive proportion in the population increases with age, and over 90% of children aged 10 years had antibody levels > 0.01 IU/ml in Myanmar in 1969, before the vaccine was introduced (Chapter 2 Figure 4) (200). The correlation between the prevalence of negative Schick results and cutaneous diphtheria incidence among children was described in Sri Lanka in the 1960s (180): both of them increased with age. The proportion of Schick-negative (immune) individuals reached 70% at 7 years of age in Sri Lanka (Chapter 2 Figure 4).

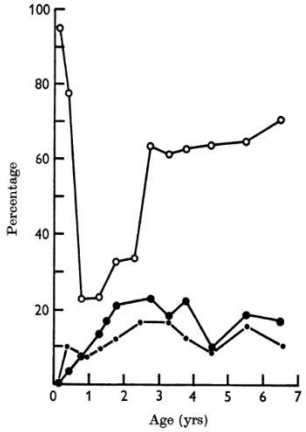
Most residents of tropical areas were reported to be Schick negative (immune), although the incidence of respiratory diphtheria was rare (200). The proportion of immune individuals among the young age group in tropical areas was higher than that in the USA in 1929, where diphtheria was endemic (201). It was also reported that individuals with diphtheria ulcers had high titres of anti-diphtheria toxoid antibodies (176, 202), although toxin-induced complications were rare with cutaneous diphtheria, which was explained by less absorption of the toxin through the skin (203). Based on these findings, cutaneous diphtheria seemed to protect individuals in tropical countries from respiratory diphtheria infection, although there was a lack of evidence to confirm this observation (176, 200).

Chapter 2 Figure 4. Seroprevalence of diphtheria by age in Myanmar in the 1960s and cutaneous diphtheria and immunity by age among children in Sri Lanka
(adapted from Gunatillake et al. 1967 (180) and Kritz et al. 1980 (200))

Age-specific seroprevalence in Myanmar, in 1969, by different cut-off values (0.01, 0.03, 0.1, 1.0 and 10.0 IU/ml)



Age-specific prevalence of Schick negative and carrier of cutaneous diphtheria



Results of Schick survey and carrier survey. Schick negative Children ○—○ Incidence of cutaneous ulcers, ●—● total isolations of *C.diphtheriae* •—•

6. Chapter Summary

Diphtheria is a severe acute respiratory disease with a high mortality rate if untreated. Diphtheria is typically caused by toxigenic *C. diphtheriae* infection, which is transmitted person-to-person by droplets.

Toxigenic *C. diphtheriae* carrying *β* corynephage with tox gene produce exotoxin, the main pathogenic agent for clinical symptoms of diphtheria. Non-toxigenic *C. diphtheriae* does not cause toxin-mediated disease in the human host and exists as part of the normal flora in human or other animal hosts or the environment.

Diphtheria toxin is not essential for the phage or its lysogenic host bacteria (i.e., *C. diphtheriae*). Yet the ability to produce toxin does have survival value for both β-phage and its bacterial host in a non-vaccinated human population. The toxin produced by toxigenic *C. diphtheriae* alters the mucous membrane of the host, which allows the bacteria to colonise and reproduce easily. Prophage incorporated within the bacteria gene is also replicated with the host bacteria. Therefore, the toxigenic strain has the advantage of surviving and proliferating compared with non-toxigenic strains in a non-vaccinated human population.

When there is a clinical case of diphtheria infected with toxigenic *C. diphtheriae*, bacteria rapidly spread through droplet infection to other human hosts associated with the case in a non-vaccinated community. In contrast, those with immunity against the toxin can harbour

the toxigenic *C. diphtheriae* for several weeks in their upper respiratory tracts. Under this condition, bacteria are transmitted to other susceptible individuals directly or via a series of healthy carriers having a protective level of anti-diphtheria toxoid antibodies if some individuals in the community were vaccinated. In a largely vaccinated population, bacterial transmission from person to person will progressively diminish. Indeed, the *tox* gene-carrying toxigenic strains have been observed to disappear from the normal flora in the upper respiratory tract of vaccinated human populations. In contrast, non-toxigenic strains without *tox* genes do not appear to be diminished in a vaccinated human population (129, 130). Therefore, the advantage of toxigenicity of the toxigenic strain compared with the non-toxigenic strain is eliminated among the well-vaccinated population.

Non-toxigenic *C. diphtheriae* can be a part of the normal flora of human hosts. When a toxigenic strain reaches the human host harbouring a non-toxigenic strain, the *tox* gene can be passed from the toxigenic strain to the non-toxigenic strain. This process is called phage-mediated lysogenic conversion, and the non-toxigenic *C. diphtheriae* is converted to the toxin-producible strain. Bacteriophage is thought to be the most abundant form of life on earth and is found in any environment (141). Therefore, there is always a potential threat that toxigenic strains will emerge among the population in which an effective vaccination programme has already eliminated toxigenic strains.

Recently, non-toxigenic *tox* gene-bearing (NTTB) strains of *C. diphtheriae* were identified in well-vaccinated communities in Europe (106, 107). NTTB strain invades the human-cell without mediation by the toxin. NTTB strain probably evolved in the environment in which vaccinated hosts are dominant and *tox*-gene does not have an advantage to the transmission of bacteria. As NTTB strains cause a different kind of disease without toxin (146), it is unclear whether the current toxoid vaccine can prevent colonisation or eliminate non-toxigenic strains from human hosts. At the same time, exposure to the NTTB strain will not increase the anti-diphtheria toxoid antibodies theoretically; therefore, the host will remain susceptible to toxigenic Corynebacteria.

Naturally acquired anti-diphtheria toxin antibody does not last for life. Protective immunity against diphtheria toxin was boosted and maintained by repeated natural infection or exposure over time in the pre-vaccination era. Therefore, the most susceptible group was young children until the vaccine was introduced.

As mentioned above, vaccine-induced protection of individuals reduces the transmission of bacteria and secondarily reduces the natural exposure in the population. In a community where natural exposure has decreased due to the child vaccination programme, individuals become susceptible when their vaccine-derived immunity wanes. Population at the age

beyond the period the last dose of vaccine could protect will become susceptible. The entire population would be protected if booster dose vaccinations were provided to the population at the appropriate intervals. Unless the entire population has protection against diphtheria toxin, diphtheria carriers will not be eliminated in the population; thereby, the transmission will continue, and the susceptible individuals remaining in the population will be infected.

Cutaneous infection of *Corynebacterium* species may play a role in maintaining transmission, and this form of infection may act as a reservoir for *C. diphtheriae* in human hosts. Cutaneous infection most likely induces immunity against diphtheria toxin and protects the host from respiratory infection, although this form of the disease is not well understood.

MLST is one of the most useful methods to identify the genetic characteristics of *Corynebacterium* species and to track transmission. TNT is a gold-standard assay to identify functional antibody levels in human sera, while ELISA is also commonly used in seroepidemiological studies.

The current DTP vaccine is one of the most effective vaccines to control bacterial disease. Although the toxoid vaccine does not prevent infection, it prevents the development of toxin-induced symptoms and death. There is no evidence that currently circulating strains in endemic areas are escaping from vaccine-mediated protection at the molecular level. The vaccine still plays a leading role in controlling diphtheria.

Chapter 3: Diphtheria outbreaks in schools in Central Highland districts in Vietnam between 2015 and 2018

Chapter overview

The aim of this chapter is to describe the epidemiological characteristics of recent diphtheria cases reported in rural Vietnam. This chapter describes the age, sex, vaccination history, and disease onset of diphtheria cases identified between 2015 and 2018 in Central Highland districts in Vietnam and neighbouring Lao PDR where the most recent diphtheria outbreaks were reported in 2015. MLST was conducted to identify the genetic information of each *C. diphtheriae* isolates detected during the investigation. This chapter raises questions about the mechanism of diphtheria incidence in the post-vaccination era, especially in LMICs that have provided three primary doses of DTP for infants for the last 30 years.

Chapter summary

The study summarises case-based information on diphtheria identified in the Central region in Vietnam. In total, 95 suspected cases and persons epidemiologically linked to confirmed cases were identified in five districts in Quang Nam province and Quang Ngai province between 2015 and 2018. The main findings and discussion of this chapter are listed below:

- A total of 22 laboratory-confirmed symptomatic cases were aged between 3 and 27 years. Only 9% of cases were younger than 5 years, and 73% were aged 5–14 years. Of the cases, 14 were male, and 8 were female.
- Of the 13 cases with a record of vaccination, 7 (53 %) had apparently not received DTP vaccine. However, two deaths (7 and 13 years old) occurred in children who had been vaccinated at least three times. Those deaths suggest that diphtheria toxoid vaccine-derived immunity might have waned, or that the vaccine might not be effective for some reasons, such as broken cold chains or low immune response in the hosts.
- The local vaccination records for ten villages in Nam Tra My district, Quang Nam province, were obtained from local authority. The administrative coverage was calculated based on the number of vaccinated individuals divided by the number of estimated population less than one year-old between 2013 and 2016. DTP3 coverage was compared between two areas: one is three villages where cases were identified (57% [95% CI: 53.3–61.2]), and another is seven surrounding villages where no cases were identified (77% [95% CI: 74.9-79.0%]) in the same district. *Chi-squared* test shows a significantly low DTP3 coverage in the area where cases were identified ($p<0.01$). Diphtheria cases are found in areas with low infant DTP3 coverage despite most of the affected cases being school-age children.

- DTP vaccination for infants was suspended in Vietnam for 6 months in 2013, which led to a sudden decline in DTP3 coverage in the entire country. This might have triggered the outbreaks.
- Schools (from nursery to high school) in the five districts have dormitories. Cases from the same school shared the same MLST type, which indicated the transmission may have occurred in school dormitories. Crowded school dormitories may be a risk factor for diphtheria transmission.
- Four MLSTs were identified in the study area, and one MLST was found in each cluster, which mainly comprised of individuals attending the same school. This observation confirmed that multiple strains were circulating in Central Vietnam, rather than the one imported strain from Lao PDR that had spread to different areas. The sparse epi-curve suggested that infection continued through asymptomatic carriers of toxigenic or non-toxigenic *C. diphtheriae*.

Based on the findings in this chapter, several research questions were proposed, which are addressed in the later chapter of this thesis.

- What is population immunity in Vietnam? (Chapters 5 and 6)
- Does Vietnam need a school-entry booster dose? If so, which age is appropriate? (Chapters 4 and 5)
- Does a school-entry booster dose prevent future outbreaks? (Chapter 6)
- What are the risk factors for diphtheria incidence? (Chapter 6)

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SECTION A – Student Details

Student ID Number	lsh422077	**Title**	Dr.
First Name(s)	Noriko		
Surname/Family Name	Kitamura		
Thesis Title	Understanding factors contributing to outbreaks of diphtheria and implications for vaccination policy in Viet Nam		
Primary Supervisor	Paul Fine		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Emerging Infectious Diseases (Kitamura N, Le TTT, Le LT, Nguyen LD, Dao AT, Hoang TT, Yoshihara K, Iijima M, The TM, Do HM, Le HX, Do HT, Dang AD, Vien MQ, Yoshida LM. Diphtheria Outbreaks in Schools in Central Highland Districts, Vietnam, 2015-2018. Emerg Infect Dis. 2020 Mar;26(3):596-600. doi: 10.3201/eid2603.191027. PMID: 32091368; PMCID: PMC7045818.)		
When was the work published?	2020 March		
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Stage of publication	Choose an item.

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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I have planned and designed a research and conducted a data collection at the local authority.

SECTION E

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Date	1 September 2022

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Date	21 September 2022

Diphtheria Outbreaks in Schools in Central Highland Districts, Vietnam, 2015–2018

Noriko Kitamura, Thao T.T. Le, Lien T. Le, Luong D. Nguyen, Anh T. Dao, Thanh T. Hoang, Keisuke Yoshihara, Makiko Iijima, Tran M. The, Hung M. Do, Huy X. Le, Hung T. Do, Anh D. Dang, Mai Q. Vien, Lay-Myint Yoshida

During 2015–2018, seven schools in rural Vietnam experienced diphtheria outbreaks. Multilocus sequence types were the same within schools but differed between schools. Low vaccine coverage and crowded dormitories might have contributed to the outbreaks. Authorities should consider administering routine vaccinations and booster doses for students entering the school system.

Diphtheria is a serious childhood disease with a high mortality rate (*1*). After a diphtheria–tetanus–pertussis vaccine (DTP) was introduced in the early 20th century, the number of cases dramatically decreased. Incidence reached a low of 4,333 cases in 2006, but more recently, the number of reported cases has increased, with incidence reaching 16,648 cases in 2018 (*2*).

In 1981, Vietnam introduced a vaccination program in which participants received 3 primary doses of DTP (DTP3) vaccine; in 2011, a booster shot (DTP4) to be given 18 months after the initial doses was added (*3*). Although diphtheria cases had become sporadic by 2010, beginning in 2013, outbreaks occurred in the western and central highland areas of Vietnam, which prompted our study (*4*).

The Study

During June 2015–April 2018, the Pasteur Institute in Nha Trang, Vietnam, and the provincial health authority investigated 46 cases involving patients with suspected diphtheria, 8 of whom died, and 49 asymptomatic contacts in the provinces of Quang Nam and Quang Ngai in the central highlands region of Vietnam (Figure 1). We used standard case investigation forms to collect demographic and clinical information. We collected throat swab specimens from 93 patients and contacts but were unable to collect samples from 2 patients who had died. No cutaneous diphtheria was reported.

We used sheep blood agar and tellurite medium cultures to identify *Corynebacterium diphtheriae* and extracted DNA with a QIAGEN DNA Mini Kit (QIAGEN, https://www.qiagen.com), following a standard protocol. We used 2 sets of primers, Tox1/Tox2 and Dipht6F/Dipht6R, for PCR testing (*5*). The Elek test for diphtheria is not available in Vietnam.

Laboratory testing confirmed diphtheria in 22 of 46 suspected cases: 17 patients, including 4 who died, tested positive in both culture and PCR tests, whereas 5 patients, including 1 who died, tested positive only by PCR. We categorized diagnosis as epidemiologic for 10 patients for whom PCR results were not available, 7 suspected cases and 3 in which the person died. We confirmed 2 of 49 asymptomatic contacts as carriers of diphtheria (*6*).

We used Api Coryne (bioMérieux, https://www.biomerieux.com) to identify biotypes of *C. diphtheriae* isolates; 15 of 17 culture-positive isolates were biotype *mitis* and 1 each was *gravis* and *intermedius*. We conducted multilocus sequence typing (MLST) by using 7 primer sets for *C. diphtheriae* housekeeping genes according to reported protocol (*7*). Using the *C. diphtheriae* MLST database (https://pubmlst.org/cdiphtheriae), we detected 4 sequence types (STs): ST67 (n = 7), ST209 (n = 9), ST243 (n = 7), and ST244 (n = 1).

Among the 31 patients with confirmed or suspected diphtheria, 21 (60%) were male; age range was 1–45 years (median 10 years). We summarized case

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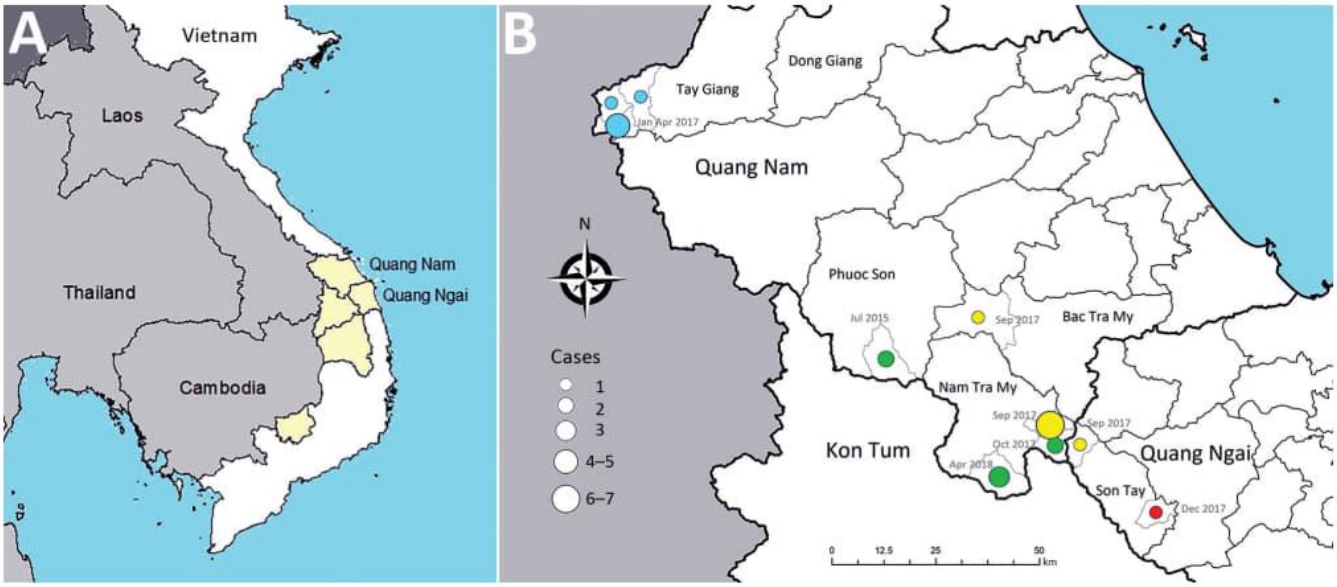

Figure 1. A) Provinces where diphtheria cases were identified in Vietnam in 2010s. Diphtheria cases were reported from provinces (shaded) neighboring Laos or Cambodia. B) Laboratory-confirmed diphtheria cases in the central highlands region of Quang Nam Province and Quang Ngai Province, central Vietnam, 2015–2018. Colored circles indicate separate outbreaks. Source: https://gadm.org/download_country_v3.html

characteristics (Table 1) and epidemiologic links and STs by cluster (Table 2). The most common symptoms recorded were fever (82%), followed by pseudomembrane and difficulty swallowing (76%).

We determined geographic areas in which cases were identified (Figure 1). Most residents in the central highlands area were in ethnic minority groups. Healthcare access is limited because of mountainous terrain and social barriers. In this area, each commune has a primary and a secondary school, but 10 communes share 1 district-level high school. All students, from primary through high school, live in dormitories during the week, and 30–50 students might live in a ≈50 m^2 room.

Table 1. Characteristics of confirmed and epidemiologically linked cases of diphtheria, central highlands of Vietnam, 2015–2018*

Characteristic	Confirmed	Epidemiologically linked	Epidemiologically linked asymptomatic carriers	Total
Age, y				
<1	0 (0)	0 (0)	0 (0)	0 (0)
1–4	2 (9)	1 (10)	1 (50)	4 (12)
5–9	7 (32)	2(20)	1 (50)	10 (29)
10–14	9 (41)	0 (0)	0 (0)	9 (26)
15–19	3 (14)	4(40)	0 (0)	7 (21)
≥20	1 (5)	3(30)	0 (0)	4 (12)
Sex				
M	14 (64)	6 (55)	1 (50)	21 (60)
F	9 (36)	4 (45)	1 (50)	14 (40)
Vaccination history, no. doses				
0	9 (41)	9 (90)	0 (0)	18 (51)
1	0 (0)	0 (0)	1 (50)	1 (3)
2	1 (5)	0 (0)	0 (0)	1 (3)
≥3	3 (14)	0 (0)	1 (50)	4 (11)
Unknown	9 (41)	1 (10)	0 (0)	11 (31)
Symptoms				
Fever	18 (81)	10 (100)	NA	28 (82)
Sore throat	15 (68)	10 (100)	NA	25 (74)
Pseudomembrane	17 (77)	9 (90)	NA	26 (76)
Difficulty swallowing	14 (64)	10 (100)	NA	26 (76)
Submandibular LN swelling	14 (64)	6 (60)	NA	20 (59)
Death	5 (23)	3 (30)	NA	8 (24)
Total no./no. persons investigated (%)	22/46 (48)	10/46 (22)	2/49 (4)	34/95 (36)

*Values are no. (%) except as indicated; total no. indicated number of patients with confirmed or suspected diphtheria or with diphtheria carrier status. LN, lymphadenopathic.

Table 2. Epidemiologic link and MLST results for 34 confirmed or epidemiologically linked case-patients with diphtheria, central highlands of Vietnam, 2015–2018*

District	Date of symptom onset	Patient age, y/sex	Epidemiologic link	Vaccine status†	Died	Culture result	PCR	ST	Biotype	Case
Phuoc Son	2015 Jun 30	26/F	Patient 1	UNK	X	‡	‡			Linked
	2015 Jun 30	18/M		UNK		–	§			Linked
	2015 Jul 4	17/F		UNK	X	–	§			Linked
	2015 Jul 4	27/M	Patient 1's husband	UNK		+	+§	67	*mitis*	Confirmed
	2015 Jul 4	16/M		UNK		–	§			Linked
	2015 Jul 5	7/M	Patient 1's son	UNK		–	§			Linked
	2015 Jul 5	20/M		UNK		–	§			Linked
	2015 Jul 8	45/M		UNK		–	§			Linked
	2015 Jul 9	1/F		UNK		–	§			Linked
	2015 Jul 14	14/M		UNK		+	+§	67	*mitis*	Confirmed
	2015 Jul 14	9/F		UNK		–	§			Linked
Tay Giang	2017 Jan 10	16/M	Tay Giang HS	UNK	X	‡	‡			Linked
	2017 Jan 10	17/M		UNK	X	+	+	243	*mitis*	Confirmed
Son Tay	2017 Mar 15	13/M		3	X	+	+	209	*mitis*	Confirmed
Tay Giang	2017 Apr 20	7/M		4	X	+	+	243	*mitis*	Confirmed
	2017 Apr 22	15/F		UNK		+	+	243	*mitis*	Confirmed
	2017 Apr 25	7/M	Gari PS	UNK		+	+	243	*mitis*	Confirmed
	2017 May 20	10/M	Patient 2 (Gari SS)	UNK		+	+	243	*mitis*	Confirmed
	2017 May 20	10/M	Gari SS	3		+	+	243	*mitis*	Confirmed
	2017 May 23	15/M	Patient 2's brother's friend	0		+	+	243	*mitis*	Confirmed
Bac Tra My	2017 Sep 5	5/F		UNK		–	+	209		Confirmed
Nam Tra My	2017 Sep 27	12/M	Tra Van SS	UNK		+	+	209	*mitis*	Confirmed
	2017 Sep 27	8/M	Tra Van PS	0	X	+	+	209	*mitis*	Confirmed
	2017 Sep 30	9/F		0		–	+	209		Confirmed
	2017 Sep 30	10/F		0		–	+	209		Confirmed
	2017 Sep 30	8/F		0		–	+	209		Confirmed
	2017 Oct 3	11/F		0		+	+	209	*mitis*	Confirmed
	2017 Oct 3	10/M		0		+	+	209	*mitis*	Confirmed
Nam Tra My	2017 Oct 8	12/F	Tra Vinh SS	UNK		–	+	67		Confirmed
	2017 Oct 12	13/M		UNK		+	+	67	*mitis*	Confirmed
Son Tay	2017 Dec 24	3/F		UNK		+	+	244	*gravis*	Confirmed
Nam Tra My	2018 Apr 17	4/M	Man Di NS	2	X	–	+	67	*intermed*	Confirmed
	2018 Apr 24	4/M		3 + 1 SIA		+	+	67		Linked
	2018 Apr 24	5/F		1 + 1 SIA		–	+	67		Linked

*Biotypes are of *Corynebacterium diphtheriae* bacteria. HS, high school; MLST, multilocus sequence typing; NS, nursery school; PS, primary school; SIA, supplemental immunization activity; SS, secondary school; ST, sequence type; UNK, unknown; –, negative; +, positive.
†The time of last vaccination was infancy or at 1 y of age according to the vaccination program in Vietnam. SIA dose was given October 30, 2017, for the last 2 case-patients.
‡Culture and PCR were not performed for these persons because their samples were not collected.
§PCR was not conducted for these 10 case-patients because this technique was not available during 2015. Stored isolates from 2 culture-positive case-patients were tested by PCR in 2017.

After January 2017, in each commune, diphtheria clusters formed mainly by school; cases in each school-based cluster shared the same ST. School clusters of the same ST in 2 communes in Tay Giang District were linked by a student who commuted between the communes. We could not identify any other epidemiologic links between clusters. An epidemic curve (Figure 2) showed the ST and outcome of cases by their onset. A long gap between clusters might indicate that the disease was transmitted through asymptomatic or skin carriers. However, further genomic testing is necessary to clarify the transmission pathway.

Of 8 persons who died, 3 were vaccinated, 1 each with 2, 3, and 4 doses. However, the vaccination history of 85% of patients was unknown. To compensate for the lack of vaccination history, we obtained administrative details of vaccination coverage in Nam Tra My District during 2013–2016. Of the 10 communes, only 3 (Tra Van, Tra Vinh, and Tra Nam) reported cases. We compared the ratios of vaccinated and unvaccinated children and found a significantly smaller proportion of children had received DTP3 in the outbreak communes than in nonoutbreak communes (57% [95% CI 53.3%–61.2%] vs. 77% [95% CI 87.0%–90.1%]; $p<0.05$ by χ^2 test).

Conclusions

Our investigation detected 22 patients with laboratory-confirmed *C. diphtheriae* cases during 2015–2018 in this region of Vietnam, 83% of whom were >5

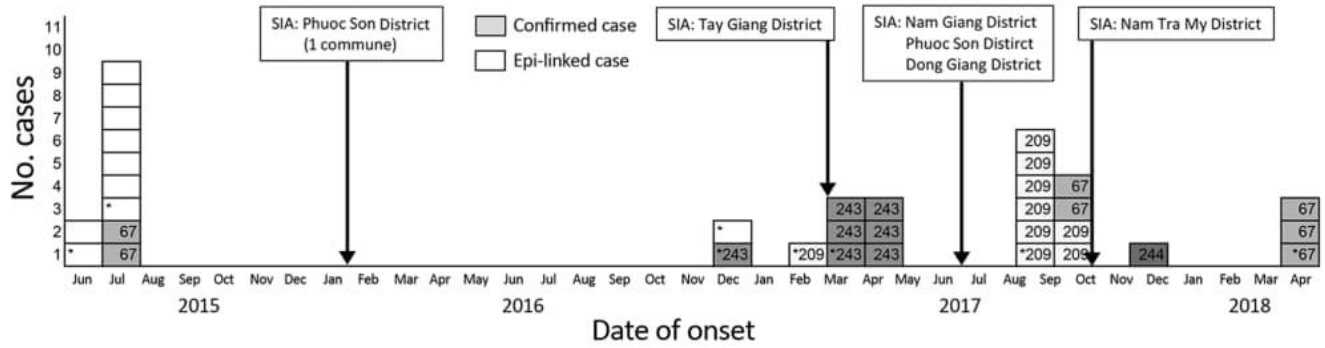

Figure 2. Confirmed and probable cases of diphtheria identified during June 2015–April 2018 in Vietnam. Numbers indicate multilocus sequencing type of confirmed cases with sequence types (STs) ST67, ST 243, ST209, and ST244 (gray shading). White indicates epidemiologically linked cases, and asterisks indicate cases in which the patient died. Epi, epidemiologically; SIA, supplemental immunization activity.

years of age. It has been predicted that age of diphtheria case-patients could increase after introduction of DTP because a high proportion of older persons will be susceptible to the disease due to reduced circulation of bacteria, especially when no booster dose is provided (*8*). The 4 MLST types identified in this study (ST67, ST209, ST243, and ST244) were also identified in Thailand, Cambodia, the Philippines, and Binh Phuoc Province in Vietnam in the 2010s (*4*,*9*,*10*). We found only 1 ST in each cluster location, which might indicate 1 person as the source of infection in each location. In addition, we identified no clear epidemiologic link among clusters. Detecting different STs between clusters indicates that multiple strains of *C. diphtheriae* were circulating in Vietnam, as well as in neighboring countries. This transmission pattern might not have changed since the prevaccination era when diphtheria was reported to spread from school to school or neighborhood to neighborhood (*11*).

The reemergence of diphtheria in Vietnam raises several concerns. Administrative coverage, although not always accurate, indicated DTP3 coverage of 57%, possibly creating a larger pool of susceptible people. In 2013, the health service temporarily suspended DTP immunization during a severe adverse event case investigation, which halved DTP3 coverage in the country (*2*) and potentially led to outbreaks. Students also share crowded school dormitories, which is a major factor for spreading disease. Moreover, students go home on weekends, increasing the chance of transmission between their schools and homes. Our finding of vaccinated people dying is particularly alarming because it might indicate a waning of vaccine-derived immunity.

Several interventions were conducted to control outbreaks. Erythromycin tablets were distributed to all contacts of diphtheria patients. However, only 2 asymptomatic carriers were identified among 49 contacts, lower than expected considering that 97% of case-patients could be asymptomatic in a vaccinated population (*12*). However, the sensitivity of laboratory testing might have been low because of the length of time required to collect and transport samples or because of prior antimicrobial drug use, so some carriers likely were not identified.

Supplemental immunization activities were conducted in the outbreak area and 2 neighboring districts (Nam Giang and Dong Giang). Healthcare agencies initiated 2 campaigns: the first, targeting persons 5–40 years of age, sought to administer 3 doses of tetanus–diphtheria vaccine and achieved >90% coverage. Simultaneously, a second campaign was conducted to administer DPT to previously unvaccinated children 1–4 years of age. However, 1 unvaccinated person with diphtheria and 2 asymptomatic carriers who had received 1 dose of DPT were reported 6 months after the supplemental immunization activity. This finding was probably because diphtheria toxoid vaccine does not prevent transmission but prevents respiratory disease (*13*); thus, carriage of the organism persists.

Although Vietnam has maintained high DTP3 coverage nationally, efforts should be intensified to increase coverage in specific areas of the country (*14*). Persistent immunity resulting from DTP3 alone is not apparent (*14*), and immunity might wane before children start school (*15*). The World Health Organization recommends that students receive a booster vaccination when entering school (*15*). However, even if this recommendation is adopted, maintaining high uptake of primary and booster doses remains critical.

Chapter 4: Waning rate of immunity and duration of protective immunity against diphtheria toxoid as a function of age and number of doses: Systematic review and quantitative data analysis

Chapter overview

This chapter aims to estimate the optimal booster dose interval, especially after three primary doses and the fourth dose given in the second year of life, as no study has estimated them to date. For estimating the optimal intervals for DTP vaccinations, a systematic review was conducted to quantify the waning rate of diphtheria immunity and the duration of protective immunity after various numbers of doses. As longitudinal studies are rare and none have targeted children, publicly available data of cross-sectional seroprevalence surveys that measured anti-diphtheria toxoid antibodies were searched in three databases. The results provided useful information to discuss optimal booster dose schedules in Vietnam or other LMICs.

Chapter summary

A systematic review identified three published articles for quantitative analysis. The three articles included national and subnational seroprevalence data stratified by single-year age collected in 15 European countries between 1995 and 2013 to quantify the waning rate of immunity and the duration of vaccine protection after a different number of vaccine doses. The data consisted of 196 single-age data points. The study analysed the obtained data by a linear regression with random-intercept model allowing the heterogeneity for different countries. The log-scaled geometric mean of concentration (GMC) of ant-diphtheria toxoid IgG in each year in each country was used as an outcome variable, assuming that the GMC declined exponentially.

The annual percentage decrease of GMC was 26% (95% CI: 20.5–31.9),17% (95% CI: 12.2–21.9) and 7% (95% CI: 1.7–11.5) per year after three, four, and five doses, respectively. The GMC was predicted to decline to 0.1 IU/ml 2.5 years (95% CI: 0.9–4.0), 10.3 years (95% CI: 7.1–13.6), and 25.1 years (95% CI: 7.6–42.6) after receiving three, four, and five doses, respectively.

There were several limitations in this analysis. The 15 countries used different types, compositions, and vaccine schedules or had different epidemiological backgrounds. Participants' vaccination history was not available; therefore, it was assumed that participants received the DTP vaccine following the recommended schedule of national immunisation programmes in each country. In addition, the vaccinated proportion of participants in each cohort was assumed to be the same as the national administrative

coverage of DTP3. Furthermore, booster dose coverage was not available in any of the countries. It was assumed to be the same as the DTP3 coverage; however, it was most likely lower than the DTP3 coverage. The single-year stratified cross-sectional data was treated similar to the cohort data followed up by the same individuals each year.

There are several strengths of this analysis. Of the 15 countries, data from 13 were collected by a large-scale national serosurvey under the Euro-Network surveillance. Therefore, their survey methods and the antibody data were standardised. Diphtheria epidemiology in Europe was different from current LMICs; however, using a well-vaccinated European population was essential to estimate the waning rate of immunity without interference from the booster effect of natural infection.

The results drawn from the study could serve as a reference for the duration of protective immunity against diphtheria and should be considered in decision-making regarding booster dose timing when booster doses are introduced in Vietnam or other countries.

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SECTION A – Student Details

Student ID Number	lsh422077	**Title**	Dr.
First Name(s)	Noriko		
Surname/Family Name	Kitamura		
Thesis Title	Understanding factors contributing to outbreaks of diphtheria and implications for vaccination policy in Viet Nam		
Primary Supervisor	Paul Fine		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Human Vaccines and Immunotherapuetics Kitamura N, Bahkali K, Chem ED, Quilty BJ, Edwards T, Toizumi M, Yoshida LM. Waning rate of immunity and duration of protective immunity against diphtheria toxoid as a function of age and number of doses: Systematic review and quantitative data analysis. Hum Vaccin Immunother. 2022 Jul 21:2099700. doi:10.1080/21645515.2022.2099700. Epub ahead of print. PMID: 35862651.		
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Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I have planned and designed a research and conducted a systematic review with others.

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REVIEW

Waning rate of immunity and duration of protective immunity against diphtheria toxoid as a function of age and number of doses: Systematic review and quantitative data analysis

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ABSTRACT

Although the burden of diphtheria has declined greatly since the introduction of vaccines, sporadic outbreaks continue to be reported. WHO recommends booster doses after a primary series, but questions remain about the optimal interval between these doses. We conducted a systematic review and quantitative data analysis to quantify the duration of protective immunity after different numbers of doses. Fifteen cross-sectional seroprevalence studies provided data on geometric mean concentration (GMC). Single-year age-stratified GMCs were analyzed using a mixed-effect linear regression model with a random intercept incorporating the between-country variability. GMC was estimated to decline to 0.1 IU/ml in 2.5 years (95% CI: 0.9–4.0), 10.3 years (95% CI: 7.1–13.6), and 25.1 years (95% CI: 7.6–42.6) after receiving three, four and five doses, respectively. The results drawn from cross-sectional data collected in countries with different epidemiologies, vaccines, and schedules had several limitations. However, these analyses contribute to the discussion of optimal timing between booster doses of diphtheria toxoid-containing vaccine.

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Antibody; diphtheria; DTP; immunity; systematic review; vaccination

Introduction

Diphtheria is an acute bacterial infectious disease caused by toxigenic *Corynebacterium diphtheriae*. The toxin, secreted by a bacteriophage, induces upper respiratory stenosis or myocarditis. The mortality rate in untreated patients is between 5% and 20%.[1] The introduction of diphtheria toxoid vaccines reduced disease incidence dramatically in all countries in the world.[2,3] However, diphtheria is still endemic in low- and middle-income countries. Multiple outbreaks, some of which were large scale, have been reported across the world in the past decade.[4–12] The incidence of diphtheria has increased more in children older than 5 years of age, than in younger children, which is thought to be due to the increasing three-dose primary series coverage and the lack of booster doses.[13]

Diphtheria toxoid vaccine was introduced into high-income countries between 1930 and 196014 and into low- and middle-income countries after 1974, as part of the Expanded Program of Immunization. It has traditionally been combined with tetanus and pertussis antigens in various formulations of diphtheria–tetanus–pertussis vaccine (DTP) and is now often combined with other antigens, for example, *Haemophilus influenzae B* (Hib), hepatitis B, and inactivated polio vaccine. The current WHO-recommended schedule of vaccination for diphtheria is three primary doses during infancy, a first booster dose between 12 and 23 months, a second booster dose between 4 and 7 years (school-entry), and a third booster dose between 9 and 14 years (school-leaving), though the optimal booster dose timing and interval remain uncertain.[15] As many low-income countries provide only three primary doses during infancy,[16] the booster dose schedule is under discussion in light of the increases in diphtheria incidence in some Asian and African countries in the past decade.[15,17–25]

In theory, the optimal timing for booster doses is determined by the waning rate of immunity and hence the duration of protective immunity against the disease after successive doses. Longitudinal data are typically more appropriate than cross-sectional data for evaluating the waning rate of immunity. Several longitudinal studies have followed up individuals' diphtheria antitoxoid antibody level over years.[26–30] However, these data were collected among adults above 20 years old. Long-term follow-up studies targeting young children are not available to the best of our knowledge. On the other hand, several cross-sectional seroprevalence data were available, so this study attempted to analyze them.

The objective of this study is to quantify waning rate and duration of protective immunity to diphtheria among children who received a three primary-dose series and each successive booster dose by using published cross-sectional survey data.

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Materials and methods

A systematic review was conducted following PRISMA guidelines to obtain data for analysis on waning immunity (PROSPERO registration number: CRD42020172475). The objective of the systematic review was to extract age-specific data on the prevalence of diphtheria antitoxoid antibodies in populations that received different numbers of DTP vaccine doses.

Search strategy for identification of studies

The electronic databases MEDLINE, EMBASE, and Global Health were screened from inception to 3 March 2020 using the following text and subject headings: ("corynebacterium" or "diphtheria") and ("vaccine*" or "vaccination" or "immuni#ation" or "schedule" or "diphtheria toxoid*") and ("seroepidemiolog*" or "seroepidemiologic studies" or "seroprevalence" or "serology" or "serological survey" or "immune adj3 status"). A manual search was conducted by screening the reference lists of the retrieved full-text articles.

Inclusion and exclusion criteria

Eligible studies were those that included data on the seroprevalence of diphtheria antitoxoid antibodies among general populations eligible for vaccination following their national immunization program. We also only included studies in which the antibody concentration was measured by the Toxin Neutralization assay (TNT) or adjusted by TNT, as the results obtained by different assays are not directly comparable.[31,32] No geographical restriction was applied.

Studies were excluded if (i) they were not published in full text, (ii) the full texts were not written in English, (iii) they did not show relevant or adequate information by full-article review, (iv) the same data were used for other eligible studies, (v) data on 1-year age-stratified immunity were not available for at least between aged 1 year and the age at which the first booster dose was scheduled, (vi) seroprevalence was not measured or adjusted by the TNT assay, (vii) the data related to migrants or refugees who had not been included in the vaccination schedule in the study setting, or (viii) the data related to immunocompromised hosts or any specific disease patients.

Study selection, data extraction, and quality assessment

Two reviewers (NK and KB) screened titles and abstracts of all studies resulting from the search after deduplication managed by Endnote X9 (Clarivate Analytics, US). After the screening, full-text articles were assessed by two reviewers (NK and KB) independently for inclusion or exclusion of each study. Two reviewers (NK and EC) extracted data from the selected studies. Some of the original antibody data were provided by the author of the original articles.

The following information were extracted: study type, publication year, study location (country), study year(s), sample size, sampling method, age (range), number or percentage of seropositive subjects, geometric mean concentration (GMC) of diphtheria antitoxoid antibody, vaccine schedule (recommended age at which vaccine should be given), vaccine coverage by year if available, and year of introduction of primary and booster dose vaccination. A booster dose was defined as any dose after the three primary doses, regardless of the primary-dose series schedule. If numerical data were not available in the full article, an online graphic tool WebPlotDigitizer was used to extract data from published graphic presentations. WebPlotDigitizer was evaluated by several articles and showed excellent consistency between the estimates from the graphics and true values and high levels of inter-coder reliability and validity.[33–35]

Assessment of risk of bias in individual studies was carried out by two investigators (NK and KB) using the tool developed by Hoy et al.[36] Each study was scored from 0 to 10, with risk determined as low (score >8), moderate (6–8), or high (≤5).

As available vaccination coverage data were limited in the original publications, the national DTP3 annual coverage levels in each country were extracted from the WHO data repository for all countries.[16] DTP3 coverage was defined as the proportion of those who completed a three primary-dose series of DTP among the population.

Assumptions about the data

GMC data by single-year age strata were extracted from the original articles and used for the data analysis. We assumed that single-year age stratified GMCs in each country were equivalent to the antibody level measured in the same individual in successive years. We assumed that GMCs increased at the age at which each vaccine dose was scheduled, and GMCs decreased by year similar to the immunity levels in individuals.

Study subjects were assumed to have been offered vaccination according to the vaccination schedule in place in their country at the time of the study. The vaccination status of each study subject was not available. Therefore, the national DTP3 coverage in the birth year of the study subjects was assumed to apply to each birth cohort in each country. Similarly, the booster dose coverage level was not available at individual or national levels, and thus all booster dose coverages were assumed to be the same as DTP3 coverage for each birth cohort. Data on individuals over 20 years of age were excluded as they were not stratified by single-year age.

Statistical analysis

GMC was calculated by exponentiating the mean log antibody level of subjects and was assumed to decrease exponentially (constantly on a log-scale) to model the waning of immunity.[28,29]

Waning immunity was investigated by analyzing GMC on a logarithmic scale. The time variable was the number of years since the age of the last scheduled vaccination. It was assumed that the waning rates of GMC were different after each successive booster doses, but were the same for all countries, and that the peak immunity levels after receiving a booster dose varied between countries. The peak immunity level might vary due to different vaccine composition or different age at which the vaccine is given. The peak immunity level may also vary by population coverage within a country. A model with variation

in peak immunity level allows for more flexibility in the analysis. Therefore, mixed-effect linear regression models with a random intercept incorporating between-country variation were used to model the waning of immunity as a function of age in the cross-sectional data.

The number of doses was included in the model as a categorical variable to adjust the peak immunity level after receiving a booster dose and to assess the modification effect on the waning rate. DTP3 coverage levels were included in the model as a continuous variable, and immunity levels in each birth cohort in each country were adjusted for DTP3 coverage. These two factors were included as a fixed effect as they were assumed to have a constant effect across all countries. The model used for the data analysis was expressed as below.

$$Y_{ij} = \beta_0 + \mu_{0i} + \beta_1 * t_{ij} + \beta_2 * d4_{ij} + \beta_3 * d5_{ij} + \beta_4 * t_{ij} * d4_{ij} + \beta_5 * t_{ij} * d5_{ij} + \beta_6 * c_{ij} + e_{ij}$$

where $Y_{ij} = \log_{10}$ GMC in country i at time j, i = individual country,

t = time since the age of last scheduled vaccine dose (year),

d = 3, 4, or 5 doses (categorical variable), c = 0% to 100% coverage (continuous variable),

μ_{0i} = random effects, e_{ij} = error,

Data analyses were conducted using STATA15 (STATA Corp LLC, College Station, TX, USA), and data visualization were conducted using R (R Core Team (2020). Vienna, Austria).

Peak immunity level, waning rate of immunity, and duration of protective immunity after each number of vaccine doses were quantified based on the above prediction model. Waning rate of immunity was assessed by annual percentage decrease of immunity, which was defined as (1 − annual change of immunity) x100%). A fixed effect of the peak $\log_{10}$ GMC (intercept of the model) and annual change of $\log_{10}$ GMC (slope of the model) were estimated for each number of doses at 90% of DTP3 coverage level with a 95% confidence interval (CI) from the model. As model used $\log_{10}$ GMC, back transformation was conducted to obtain the predicted peak immunity and annual change of immunity.

Duration of protective immunity was assessed as the period over which immunity (GMC) was estimated to decline to the protective threshold. The times at which GMC was predicted to decline to two standard protective thresholds (0.1 IU/ml and 0.01 IU/ml)[32] were estimated from the line of best fit assuming that DTP3 coverage was always 90%, which WHO recommends to reach. This predicted time can be considered a measure of duration of protective immunity. Duration of protective immunity is determined by peak immunity level (intercept) and waning rate (slope) after receiving each dose. Figure 1 provides a schematic image showing the hypothesized pattern of peak immunity levels and waning rates after receiving booster doses. The Delta method was used to estimate a 95% CI for the duration of protective immunity.[37,38]

Results

Systematic review

A total of 1,209 articles were identified on the electronic databases by the search strategy, and 12 articles were identified manually. After removing duplicates, 883 studies were screened. According to the eligibility criteria, 663 articles were excluded, leaving 220 eligible articles. Full articles were examined, and three articles were retained for data analysis.[39–41]

GMC data from 15 countries (Czech Republic, Denmark, Finland, France, Hungary, Ireland, Israel, Italy, Latvia, Luxembourg, Norway, Russia, Slovakia, Sweden, and the United Kingdom) were included in three articles. These 15 countries were included in the quantitative analysis of waning rate of immunity (Figure 2).

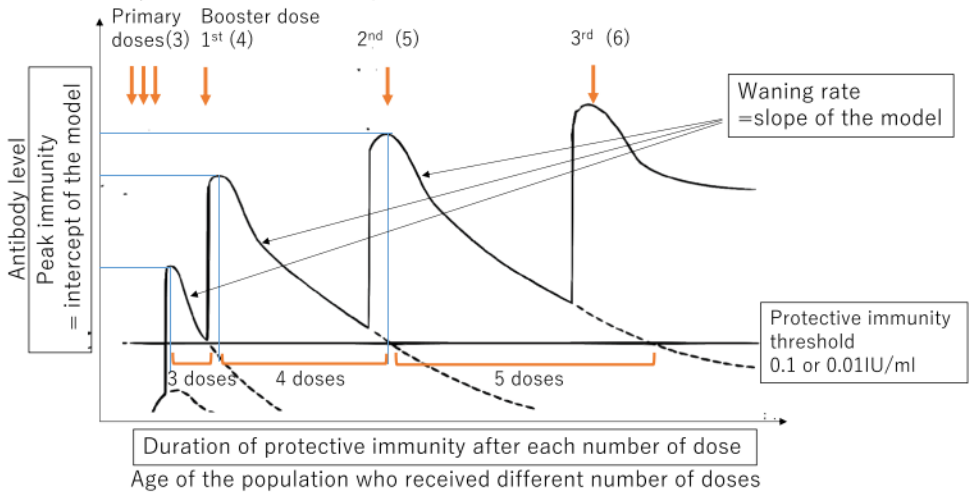

Figure 1. A hypothesized schematic image of the data and analysis and measurement of waning rate of immunity and duration of protective immunity. The peak of the immunity curve shows the peak immunity after vaccination. The slope of the graph is waning of immunity after the respective number of doses previously given. Durations of protective immunity are determined by the peak immunity levels after vaccination and waning rate (slope). Figure was adapted from "WHO immunological basis for immunization series: module 3 tetanus".[51]

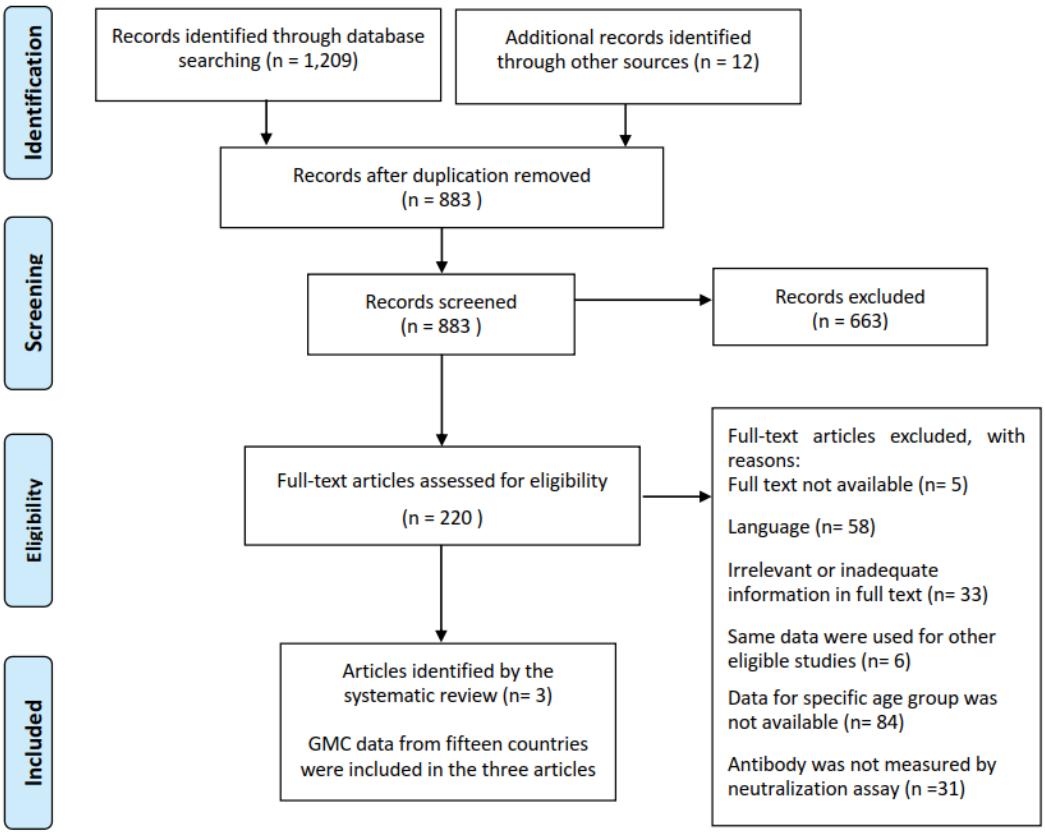

Figure 2. Results of literature search and flow diagram.

All countries were in the European region. Data from the 15 above-listed countries were collected as cross-sectional seroprevalence studies between 1995 and 2003. In the Czech Republic, Latvia, Luxembourg, Slovakia, and Sweden, serum samples were collected in population-based surveys. Other countries used residual sera collected during routine laboratory testing. All samples, except for Norway and Russia, were collected as a national serosurvey. Their samples were collected from a wide range of geographical locations within each country, and, to avoid systematic bias, sera likely not to be representative of the population were excluded (e.g. immunocompromised host).[40,41] In Norway and Russia, the samples were collected at subnational regions.[39] According to the quality assessment within studies based on Hoy's criteria, six countries (40%) had a low risk, nine (60%) had a moderate risk, and no study had a high risk of bias (Table 1).

Data from the 15 countries, including the target population, sample size, vaccination schedule, and introduction year of the booster doses, are summarized in Table 1. Age at first booster dose varied from 12 months to 10 years, and the total number of doses varied from four to seven. As the number of countries providing more than five doses of DTP was limited, we quantified waning rate of immunity and duration of protective immunity after three, four, and five doses only. The available data for analysis, such as age range and sample sizes in each country, are summarized in Table 2.

Waning of immunity and duration of protective immunity

Before conducting the analysis, all the GMC data were plotted by birth cohorts in each country. Some birth cohorts were excluded because they were over the target age of the booster doses when those doses were introduced. The antibody level was expected to reach a peak within a year of each scheduled dose. However, some delayed peaks were observed 1 to 2 years after the scheduled booster dose age. As delayed peaks affect the waning rate, data before the peak were removed. Figure 3 shows four countries' original data and how the data were treated before analysis in the regression model.

GMCs in the 15 countries were plotted by year since the age of last scheduled vaccination separately by number of doses. There was some heterogeneity in waning rate by country expressed as a slope of simple linear regression (Figure 4).

The average peak GMC level in 15 countries was expressed as an intercept predicted by the mixed-effect linear regression model. The average waning rate of GMC was expressed as a slope predicted by the model. GMC declined significantly by year after the last scheduled primary and booster dose (*p-value <0.01*). The peak GMC levels were 0.21 IU/ml, 0.71 IU/ml, and 0.58 IU/ml; the annual percentage decrease of GMC was 26%, 17%, and 7% per year, respectively, after three, four, and five doses. GMC was predicted to decline to 0.1 IU/ml in 2.5 years, 10.3 years, and 25.1 years and predicted to decline to 0.01 IU/ml in 10.0 years, 22.5 years, and 58.0 years, respectively, after receiving three, four and five doses (Table 3).

Table 1. Summary table of 15 countries included in the quantitative analysis.

Authors	Country	Study year	Age (year)	3 doses (month)	4th dose (year)	5th dose (year)	DTP intro	Booster intro*	Sample size	Sampling method	Hoy's criteria	DTP3 coverage mean (range)
di Giovine et al.	Czech Rep	2001	0-75+	2,3,4	1.5 (D)	5 (D)	1960	1986	3123	Community	10	100% (96–100%)
Edmunds et al.[31]	Denmark	1995	0-75+	3,5,12	5 (D)		1930s	1996	2989	Residual sera	8	89% (86–91%)
Edmunds et al.[31]	Finland	1995	0-75+	3,4,5	2 (D)	11-13 (d)	1943	1989*	3381	Residual sera	10	95% (90–99%)
Edmunds et al.[31]	France	1995	0-75+	2,3,4	1.5 (D)	5 (D)	1938	NA	2462	Residual sera	8	91% (79–96%)
di Giovine et al.[32]	Hungary	2003	0-60+	3,4,5	3 (D)	6 (D)	1960	1971	2600	Residual sera	8	99% (99–100%)
di Giovine et al.[32]	Ireland	2003	0-60+	2,4,6	4 (D)	12 (d)	1960	1996*	3300	Residual sera	8	66% (36–86%)
di Giovine et al.[32]	Israel	2000	0-60+	2,4,6	1 (D)	7 (D)	1951	1999	3300	Residual sera	10	92% (91–96%)
Edmunds et al.[31]	Italy	1996	0-75+	3,5,11	5-6 (D)	X (d)	1939	NA	3111	Residual sera	8	93% (83–95%)
di Giovine et al.[32]	Latvia	2003	0-60+	3,4.5,6	1.5 (D)	7 (D)	1960	1998*	3300	Community	10	91% (80–98%)
di Giovine et al.[32]	Luxembourg	2001	4-70+	2,4,5	1(D)	5 (D)	1960	1999	3200	Community	8	87% (68–98%)
Skogen et al.[30]	Norway	1994	1-12	3,5,11	11(d)		1942	NA	400	Residual sera (subnational)	6	88% (80–98%)
Skogen et al.[30]	Russia	1994	1-10	3,4.5,6	2 (D)	6 (D)	1958	NA	264	Residual sera (subnational)	6	76% (73–79%)
di Giovine et al.[32]	Slovakia	2003	0-60+	2,4,10	2 (D)	5 (D)	1960	1998	3300	Community	10	99% (99–100%)
Edmunds et al.[31]	Sweden	1995	0-75+	3,5,11	10 (D)		1951	NA	3633	Community	10	99% (99–99%)
Edmunds et al.[31]	UK	1996	0-75+	2,3,4	3.3 (D)	15 (d)	1940	1994*	3224	Residual sera	8	69% (41–94%)

X: Decennial booster dose.
D and d refer to high and low dose of diphtheria toxoid, respectively.
†Final sample size was not reported for some studies (Hungary, Ireland, Israel, Latvia, Luxembourg and Slovakia). For those studies, the target sampling size was used for sample size.
*Multiple booster doses were used in the majority of countries. The introduction years of the booster dose were for the fifth dose.
§Hoy's criterion score were used to assess the quality of the prevalence study for the systematic review.

Table 2. Age ranges and original sample sizes for the 15 countries population included in the quantitative analysis for waning rate of immunity. [30–32]

Country		After 3 doses	After 4 doses	After 5 doses	After 6 doses
Czech Republic	Age range (year)	1	2-4	7-19	ns
	Sample size	56	283	1289	
Denmark	Age range (year)	2-4	6-16	ns	ns
	Sample size	287	783		
Finland	Age range (year)	1	2-10	13-17	ns
	Sample size	100	854	470	
France	Age range (year)	1	2-5	7-10	12-15
	Sample size	45	200	173	173
Hungary	Age range (year)	ns	3-5	6-10	13-14
	Sample size		300	500	200
Ireland	Age range (year)	ns	4-9	13-19	ns
	Sample size		600	700	
Israel	Age range (year)	ns	1-6	ns	ns
	Sample size		600		
Italy	Age range (year)	1-4	8-15	ns	ns
	Sample size	359	695		
Latvia	Age range (year)	ns	2-6	8-12	14-19
	Sample size		500	500	500
Luxembourg	Age range (year)	ns	4	ns	9-11
	Sample size		100		300
Norway	Age range (year)	1-10	12	ns	ns
	Sample size	336	29		
Russia	Age range (year)	1	3-5	6-8	9-10
	Sample size	15	99	79	58
Slovakia	Age range (year)	1	3-4	6-10	ns
	Sample size	100	200	500	
Sweden	Age range (year)	1-9	10-19	ns	ns
	Sample size	528	724		
UK	Age range (year)	ns	6-13	15-17	ns
	Sample size		763	299	

ns: no samples were included in the analysis for respective countries and doses. The sample size in this table for Ireland, Israel, Hungary, Latvia, Luxembourg, and Slovakia were the number of samples that were planned to be collected as final sample sizes were not available in the cited publications.

Discussion

In this study, we quantified the peak immunity and waning rate of anti-diphtheria antitoxoid antibodies after different numbers of DTP doses. We also estimated duration of protective immunity. The prediction was conducted using 15 countries' data in Europe. The estimated duration of protective immunity may be considered an optimal booster dose interval and will be useful for countries where additional booster doses need to be introduced. Our study found that GMC was estimated to decline to 0.1 IU/ml in 2.5 years after three-dose primary series. This indicates that the currently recommended first booster dose at 12–23 months of age is reasonable.[42] In addition, GMC remained above 0.1 IU/ml in 10.3 years after four doses. This result justifies DTP schedules in some countries, such as Finland, the United Kingdom, and Japan, which provides the fifth dose about 10 years after the fourth dose. In this study, GMC remained above 0.1 IU/ml for 25.1 years after five doses, which were completed between age five and fifteen. Although our study did not measure the duration of protective immunity after the sixth dose, 25.1 years of protection is similar to a previously estimated duration of protection after six doses of DTP. A cross-sectional seroprevalence study in the Netherlands, which provides a sixth dose of DTP at 9 years of age, estimated that individuals would be protected until they were 37 years old.[43] While WHO anticipated more than six doses would not be required in many populations,[44] it is still unclear whether additional doses are required for the middle-aged population.

We previously measured the duration of protective immunity using 2-year cohort data in a well-vaccinated community in Vietnam.[45] This study showed that IgG remains above 0.1 IU/ml for 4.3 years (95%CI:3.5,5.3) after the fourth dose of DTP was given at 18 months of age. This result supports the recommendation for a school-entry booster dose. A cross-sectional seroprevalence study in South India showed that the proportion of children whose IgG levels were above 0.1 IU/ml declined from 47.4% at age five to 12.6% at age 17 after fifth dose of vaccine was given at age five.[46] Indian data showed much faster waning rate than estimated in this review. The reason for this difference might be low vaccination coverage in older age groups in the Indian population. Truelove et al. conducted a systematic review and pooled analysis to estimate waning rate of immunity by using a mixed-effect log-linear regression model.[47] This study analyzed cross-sectional seroprevalence data with 888 age group observations from 62 studies. The original studies were conducted in Europe, Asia, and North and South America between 1962 and 2016. Their estimated annual decline of proportion of immune (above 0.1IU/ml) by age since vaccination of DTP was 0.75% per year of age (95% CI, 0.25–1.24%). We have estimated the annual decline of proportion of immune (data not shown) along with waning rate of GMC levels, but our results indicated much more rapid decline than their estimate. Potential reasons for the difference include that their analysis combined serological data measured by different assays (i.e. ELISA and TNT), different age groups, different vaccine doses previously given, different study periods, and different geographical areas. Discrepancies between the results obtained from Asia and Europe cannot be explained by single factors and are probably attributable to multiple epidemiological differences between regions or variation of source data and estimation methods.

There are several limitations to this analysis. There are differences between countries in terms of vaccine composition and schedule, vaccination coverage, (Table 1) background diphtheria incidence, and the original type of serological assays, while we assumed waning rates were the same in all the countries analyzed. Ideally, these additional factors affecting immunity should be adjusted but it was not possible to quantify them, except for the vaccination coverage. The vaccination status of the study populations was not available and might have been different from the national coverage. Further, data on booster dose coverage were also not available, which may have a significant influence on immunity in later life. It was assumed that the booster dose coverage was the same as DTP3 in each birth cohort, although this coverage is likely to be lower than DTP3. This assumption may have led to an underestimation of the duration of protective immunity if all doses were actually received. DTP3 coverage did not modify the GMC level in our analysis; however, this might be because unadjusted factors, mentioned above, masked the effect of coverage. The average peak immunity after the fifth dose were not increased from the fourth dose. This might have occurred because of low fifth dose coverage, delayed timing

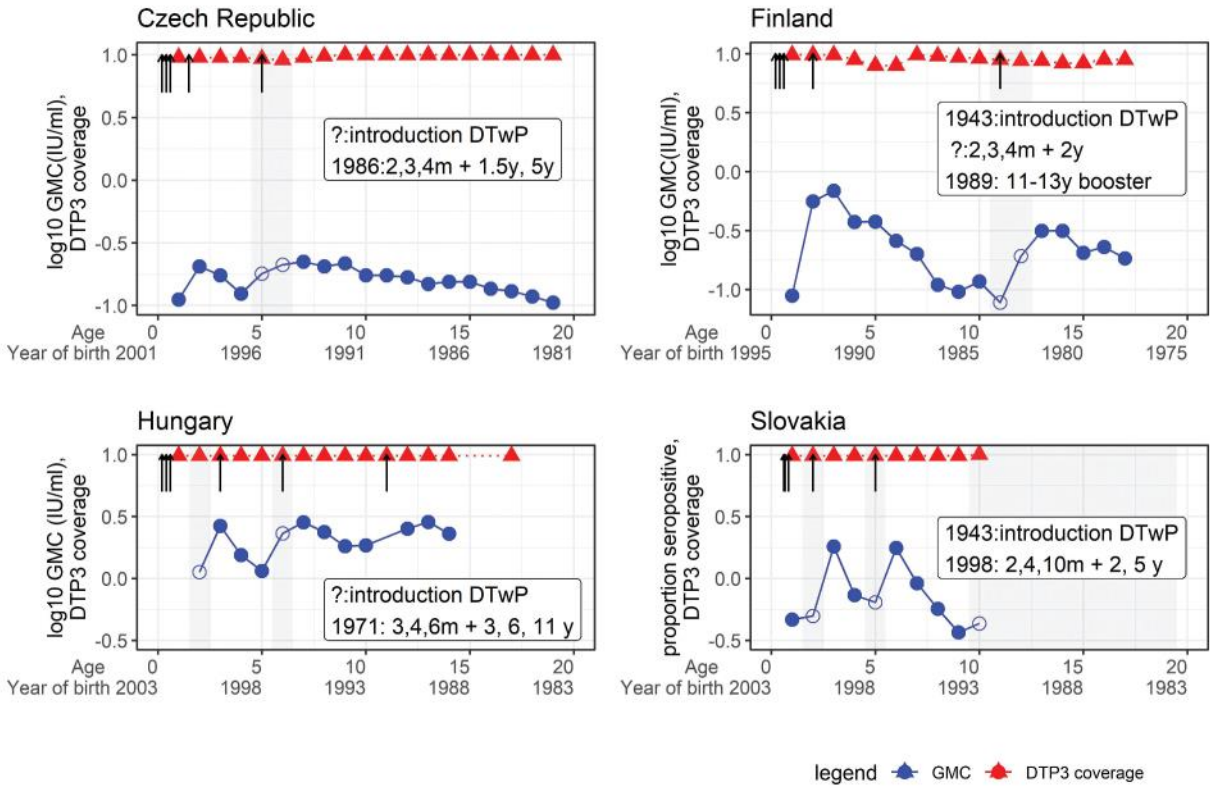

Figure 3. Seroprevalence and DTP3 coverage by age and year of birth in data included in the analyses. These figures are a descriptive aid to show which data were used for the regression analysis. Data from four countries are shown here. Some birth cohorts shaded in gray were removed for various reasons: (1) some birth cohorts were too old to receive booster doses when they were introduced, e.g., ≥10 years old birth cohort in Slovakia, and (2) GMC was lower than the peak at the age of the scheduled booster dose, e.g., the birth cohort aged 5-6 years in the Czech Republic and the birth cohorts aged 11 and 12 years in Finland. The birth cohorts removed from the analysis were expressed as hollow circles and the remaining cohorts included in the analysis were shown as solid circles. Solid circles indicate GMC, and triangles indicate national DTP3 coverage. Arrows indicate the vaccination schedule in each country.

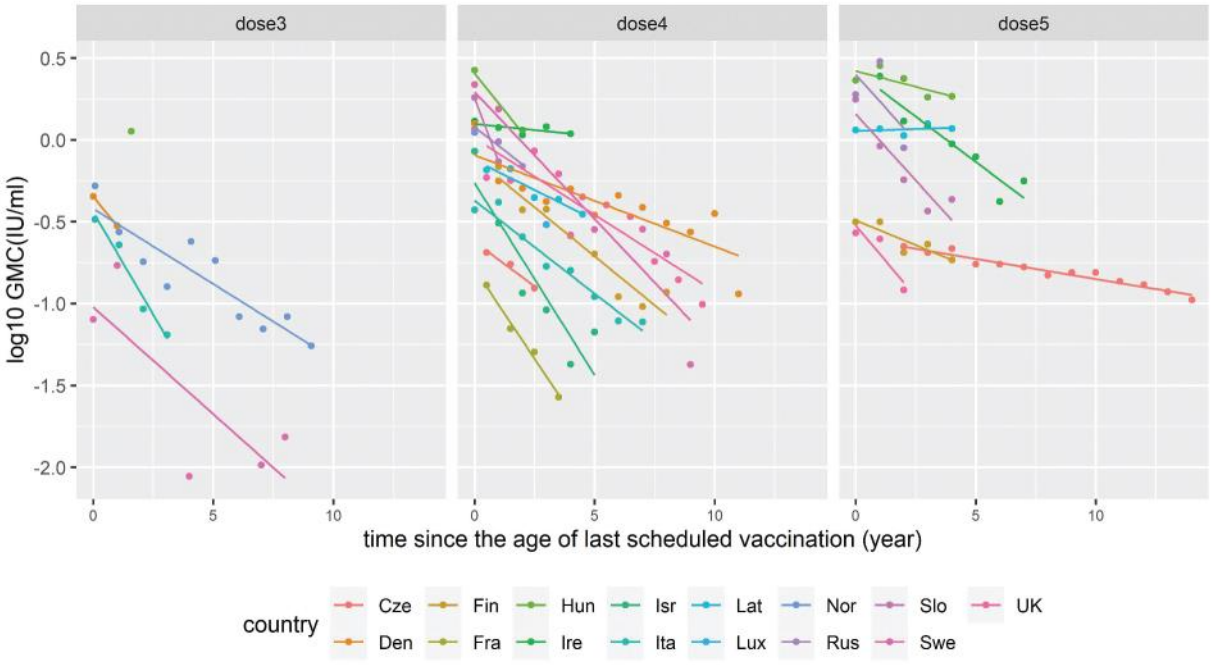

Figure 4. Declining trend of GMC over time after different numbers of DTP doses were given in 15 countries: Czech Republic, Denmark, Finland, France, Hungary, Ireland, Israel, Italy, Latvia, Luxembourg, Norway, Russia, Slovakia, Sweden, and the United Kingdom. Each observed value of GMC was plotted over time for each country on a $\log_{10}$ scale. Predicted lines were drawn by the simple linear regression of immunity over time but not adjusted for coverage.

Table 3. The peak GMC level, annual percentage decrease of GMC level, and duration of protective immunity after three, four, and five doses: Duration of protective immunity was estimated as the time at which GMC declined to 0.1 IU/ml or 0.01 IU/ml.

	Peak GMC level (intercept)		Annual percent decrease (slope)		Duration of protective immunity (intercept-threshold/slope)			
					Declined to 0.1 IU/ml		Declined to 0.01 IU/ml	
	IU/ml	(95% CI)	%	(95% CI)	year	(95% CI)	year	(95% CI)
After 3 doses	0.21	(0.13, 0.35)	26%	(20.5, 31.9%)	2.5	(0.9, 4.0)	10.0	(7.4, 12.5)
After 4 doses	0.71	(0.45, 1.12)	17%	(12.2, 21.9%)	10.3	(7.1, 13.6)	22.5	(16.1, 29.0)
After 5 doses	0.58	(0.36, 0.94)	7%	(1.7, 11.5%)	25.1	(7.6, 42.6)	58.0	(16.6, 99.4)

*Peak GMC level (intercept) and annual percentage decrease (slope) after each vaccine dose were estimated assuming at 90% of DTP3 coverage with 95% CI from the model. The mean peak GMC level (IU/ml) was calculated by 10exp("intercept"). Annual percentage decrease was calculated by (1 − 10exp("slope")) x 100%).

of vaccination, or low immune response after long interval from the fourth dose. The wide 95% CI of the predicted waning rate and that of the predicted duration of protective immunity, especially after fifth dose, are attributable to the heterogeneity by country and the limited sample size. Therefore, we cannot make firm conclusions from the current results.

As an additional limitation, it has been suggested that cutaneous diphtheria may play a role in maintaining protective immunity to diphtheria, particularly in tropical countries.[48] Since 1997, the Hib vaccine has been combined with DTP and used worldwide.[49] Modified diphtheria toxoid is used as a protein carrier in conjugate Hib vaccine, which has been shown to increase the diphtheria antitoxoid antibody level among recipients.[50] Therefore, the results derived from European data collected between 1995 and 2003, with relatively high infant vaccination coverage and homogeneous populations in temperate climate, might not be generalizable to the current populations in tropical settings. The study also has several strengths. The currently available data were searched by systematic review. National seroprevalence surveys with a large sample size were used for this analysis. The risk of bias of the source data was confirmed as low or moderate according to the Hoy's assessment criteria. Except for Norway and Russia, sampling methods were quite similar in all countries as original surveys were conducted as a multi-country study in Euro-Surveillance Network. We estimated the waning rate of vaccine-derived immunity by the number of vaccine doses, which does not appear to have been reported before. The method using already available cross-sectional serology data is simpler and cheaper than carrying out a longitudinal study to provide additional information for the vaccination schedule, notwithstanding the several limitations mentioned above.

Conclusions

We estimated the waning rate of immunity and duration of protective immunity after consecutive doses of DTP from cross-sectional seroepidemiological data with the assumption that the study participants were vaccinated according to the reported vaccination coverage. Our results indicate potential optimal booster dose intervals for diphtheria toxoid-contained vaccine. However, the several assumptions made in the method increased the risk of inaccuracy; therefore, the conclusions drawn here need to be treated cautiously. The results should be taken into consideration along with the various factors that determine appropriate vaccination schedules, including waning of other co-administered vaccine components, especially pertussis, and the epidemiological background in each country.

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Disclosure statement

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Author contributions

NK designed and conducted the systematic review, conducted the quantitative analysis, and drafted the manuscript.

NK and KB equally contributed to screening articles and investigating the quality assessment of included studies.

NK and EC equally contributed to extracting data from included studies.

BJQ and TE supported the statistical data analysis, contributed to interpreting the data, and revision of the draft.

MT and LMY contributed to interpreting the data and revision of the draft.

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Chapter 5: The seroprevalence, waning rate, and protective duration of diphtheria toxoid vaccine-derived immunity in Nha Trang, Vietnam

Chapter overview

Investigation of the recent outbreaks in Vietnam revealed that 73% of laboratory-confirmed cases were 5–14-year-old children (Chapter 3). This result suggested the immunity against diphtheria may be low among school-age children in Vietnam. This chapter aims to measure current population immunity in a well-vaccinated community in Vietnam using cross-sectional survey data. Furthermore, the study aims to estimate the waning rate of immunity against diphtheria and to estimate the duration of protection of vaccine-derived immunity using longitudinal data from two cross-sectional surveys conducted 2 years apart.

The Vietnamese MoH has been discussing the introduction of school-entry booster dose as of 2018. This chapter aims to obtain the evidence to discuss whether a booster dose is necessary and the age of optimal booster dose timing in a country where only one booster dose at 18 months of age was introduced in addition to three primary doses.

Chapter summary

An age-stratified cross-sectional seroprevalence survey was conducted in Nha Trang City, Vietnam, where their administrative DTP3 coverage was over 90%, except in 2013, and no diphtheria cases were reported for in the last decade. Seroprevalence was defined as the proportion of individuals with serum anti-diphtheria toxoid antibody ≥ 0.1 IU/ml.

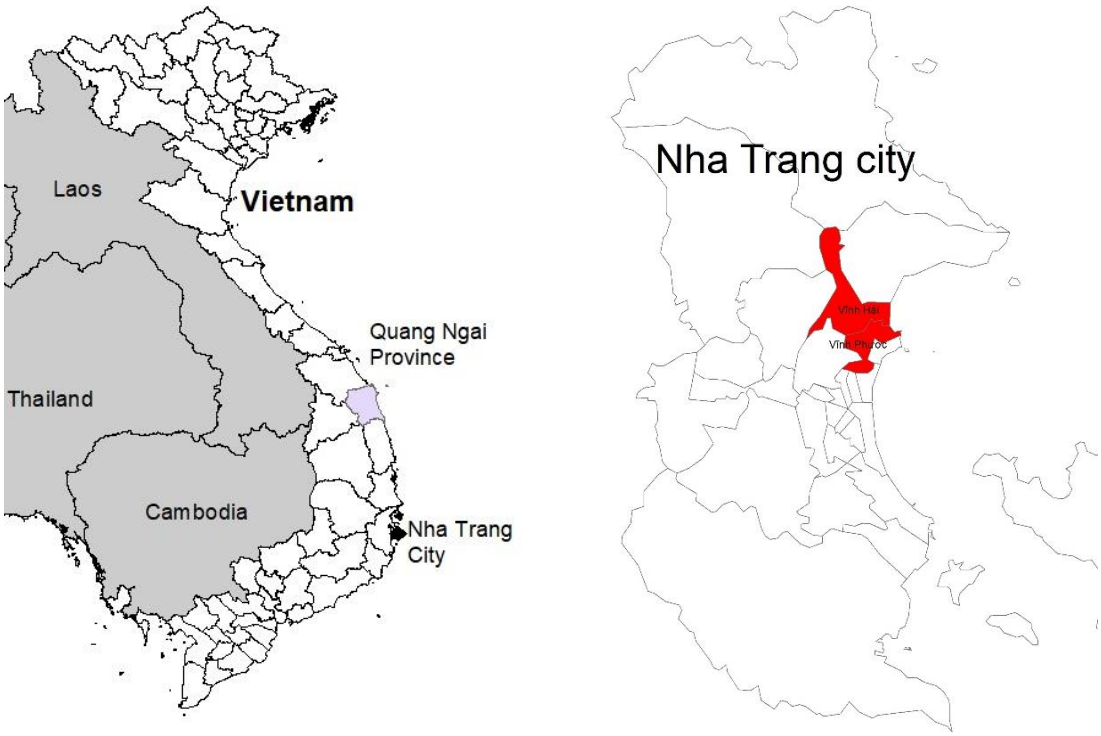
The overall weighted seroprevalence was 26% (95% CI:22–30%) in the population aged 6months to 55 years; the weighted seroprevalence for male was 25% (95% CI: 20–31%) and for female was 27% (95% CI: 22–33%). Age-specific seroprevalence was 68% (95 %CI: 52–80%), 7% (95% CI: 2–14%), 12% (95% CI: 6–20%), 33% (95% CI: 24–43%), and 28% (95% CI: 22–35%) for 0–5 years, 6–15 years, 16–25 years, 26–35 years and 36–55 years. The age-stratified seroprevalence revealed that a large proportion of the population was susceptible, especially school-age children. The results clearly suggested that the recent outbreak occurred due to low immunity in a well-vaccinated population. Therefore, an additional booster dose would be essential to maintaining protective immunity in this population.

The average level of anti-diphtheria toxoid IgG of the children aged 6 years or younger who received four doses of DTP declined by 47% (95% CI: 31–59) in 2 years. Following the mixed-effect linear regression analysis, IgG levels were estimated to be maintained above 0.1 IU/ml for 4.3 years (95% CI: 3.5–5.3) among children aged 6 years or younger who had

received four doses of DTP. Considering that the last dose is given at 18 months of age in Vietnam, the second booster dose is recommended between 5 and 7 years, which is also practical as primary school starts at 6 years of age in Vietnam.

According to the results of a WHO systematic review in 2017, vaccine-derived anti-diphtheria toxoid antibody does not wane for 10 years after the last primary dose. This chapter showed that the anti-diphtheria toxoid antibodies waned much faster than the reported rate. A school-entry booster dose will be essential to maintaining adequate protection levels in the Vietnamese population.

Chapter 5 Figure 1. The Map of Vietnam and two study communes in Nha Trang city (red)



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RESEARCH PAPER COVER SHEET

Please note that a cover sheet must be completed for each research paper included within a thesis.

SECTION A – Student Details

Student ID Number	lsh422077	**Title**	Dr.
First Name(s)	Noriko		
Surname/Family Name	Kitamura		
Thesis Title	Understanding factors contributing to outbreaks of diphtheria and implications for vaccination policy in Viet Nam		
Primary Supervisor	Paul Fine		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	International Journal of Infectious Diseases Noriko Kitamura , Lien Thuy Le , Thao Thi Thu Le , Hien-Anh Thi Nguyen , Tansy Edwards , Lina Madaniyazi , Minh Xuan Bui , Hung Thai Do , Duc-Anh Dang , Michiko Toizumi , Paul Fine , Lay-Myint Yoshida , The seroprevalence, waning rate, and protective duration of anti-diphtheria toxoid IgG antibody in Nha Trang, Vietnam, International Journal of Infectious Diseases (2022), doi: https://doi.org/10.1016/j.ijid.2022.01.025		
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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I have planned and designed a research and conducted a systematic review with others.

SECTION E

Student Signature	Noriko Kitamura [redacted]
Date	1 Sep 2022

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Date	2 September 2022

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The seroprevalence, waning rate, and protective duration of anti-diphtheria toxoid IgG antibody in Nha Trang, Vietnam

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ABSTRACT

Background: Diphtheria cases reported in Central Vietnam since 2013 were mainly in children aged 6-15 years, which may reflect an immunity gap. There is little information on population immunity against diphtheria in countries without a school-entry booster dose. We aimed to measure the age-stratified seroprevalence of anti-diphtheria toxoid antibodies, quantify the change in antibody levels in individuals over time, and estimate the length of protective immunity after vaccination in well-vaccinated communities in Vietnam.

Methods: An age-stratified seroprevalence survey among individuals aged 0-55 years was conducted at Nha Trang, Vietnam. The same participants were followed up after two years to quantify the change in antibody levels. IgG was measured using ELISA. The length of protective immunity after vaccination was estimated using a mixed-effect linear regression model with random intercept.

Results: Overall seroprevalence was 26% (95%CI:20-32%). Age-stratified seroprevalence was 68% (95%CI:4-11%), 7% (95%CI:4-11%), 12% (95%CI:7-19%), 33% (95%CI:27-40%), and 28% (95%CI:17-43%) among those aged ≤5, 6-15,16-25, 26-35, and 36-55 years, respectively. The antibody levels declined by 47% (95%CI:31-59%) over two years, and the predicted duration of vaccine-derived protective immunity after receiving four doses was 4.3 years (95%CI:3.5–5.3) among participants aged six years or younger.

Conclusion: Given the low seroprevalence and short period of vaccine protection, a school-entry booster dose (5-7 years) is recommended in Vietnam.

Background

Diphtheria is an acute infectious disease typically affecting the upper respiratory tract, which is mainly caused by toxin-producing *Corynebacterium diphtheriae*. Diphtheria used to be a major cause of child mortality, but introducing a highly effective toxoid vaccine decreased the disease burden. Recently, large-scale outbreaks were observed in Venezuela, Yemen, and the Rohingya population in Bangladesh, where the routine infant vaccination program was disrupted. Diphtheria remains endemic in many parts of the world. The number of cases increased, in particular, in South and South-

Abbreviations: DTP, the diphtheria-tetanus-pertussis; GMC, geometric mean concentration; ELISA, enzyme-linked immunosorbent assay; WHO, the World Health Organization.

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east Asia in the latter half of the 2010s compared with the earlier half (WHO 2017, Clarke et al. 2019).

Currently, WHO recommends that a three-dose primary series of diphtheria toxoid-containing vaccine should be administered in early infancy, a first booster dose in the second year of life, and a second and a third booster at the age of school entry and school leaving, respectively (WHO 2017). Nevertheless, many low-income countries have not introduced any booster dose yet. There is little information on the waning and the duration of immunity among children who receive three or four doses.

In Vietnam, the diphtheria-tetanus-pertussis (DTP) vaccine was introduced in 1981 and was replaced by the Pentavalent vaccine (DTP-Hib-HepB) in 2011 (MOH Vietnam 2012). The primary dose series was given at 2-3-4 months, and in 2012, a booster dose at 18 months was introduced (MOH Vietnam 2012). Although the number of reported cases decreased sharply from 3500 per year in 1983 to nearly zero in 2010 (Jit et al. 2015), several outbreaks have been observed since 2013.

The 76 confirmed diphtheria incidences in the Central and Highland region of Vietnam between 2014 and 2019 were in patients aged between 1 and 55 years, with 66% of the cases being children aged between 6-15 years (data in the Pasteur Institute in Nha Trang and the Institute of Hygiene and Epidemiology of Tay Nguyen, Vietnam). The age distribution in diphtheria cases reflects the pattern of population immunity and, thus, the history of DTP vaccination schedule and coverage (Galazka and Keja 1988, Galazka and Robertson 1995, Zakrzewska et al. 1997, Clarke et al. 2019). The population immunity has changed over time since the introduction of vaccine (Galazka 2000). In addition, it is known that the age at infection in diphtheria cases typically shifts to older ages after the introduction of vaccine (Galazka and Keja 1988). This raises the question of how population immunity against diphtheria is in Vietnam currently.

This study aimed to measure the age-stratified seroprevalence and geometric mean concentration (GMC) of anti-diphtheria toxoid antibodies in a study population in Nha Trang city, Vietnam, where high vaccination coverage was reported in most of the last ten years. The study also quantified the change in anti-diphtheria toxoid antibody levels as a function of age, baseline IgG level, and number of DTP vaccine doses to investigate the antibody waning pattern. Finally, the study estimated the length of time that protective immunity of >0.1 IU/ml was maintained after vaccination.

Materials and methods

Study population and area

Nha Trang city, the capital of Khanh Hoa Province in Central Vietnam, has a population of >300,000 (Yoshida et al. 2014). The two study communes, Vinh Hai and Vinh Phuoc communes, are adjacent to each other in the urban area of Nha Trang. According to a census conducted in 2015, the population size of the two communes was 42,397. Each commune has one health center, which provides essential health services, including vaccination.

Study design and sampling method

A cohort study was conducted between 2017 and 2019. The sample population was originally selected for a cross-sectional seroprevalence survey for antibodies against dengue in June 2017 (Biggs et al. 2020). The original target sample size was 500, and the participants were over-recruited, considering potential non-respondents. Finally, a total of 510 samples were collected in 2017. Age-stratified simple random sampling was conducted in each commune on the basis of census data. The five age strata were 0-5 years, 6-15 years, 16-25 years, 26-35 years, and 36-55 years in 2017, and samples of 50 were drawn randomly from each age stratum from the population data of each of the two communes. These data were available for this study, and all participants in the first cross-sectional survey were invited to join a follow-up survey in May 2019.

Study teams visited participants at their homes. Written informed consent was obtained from each participant or guardian if the participant was younger than 16 years. The survey teams interviewed each participant using a standardized questionnaire collecting information on sex, date of birth, and oral vaccination history. In addition, venous blood samples were collected from participants at the commune health centers. Ethical approval was obtained from the Vietnamese Ministry of Health and the London School of Hygiene and Tropical Medicine ethical review boards (IRB-VN01057-27/2015, LSHTM Ethics ref: 17518/17913).

Vaccination record of individuals and vaccination coverage

We obtained written vaccination records from each individual's vaccination card, in addition to the oral information, during the survey in 2019. If the vaccination card was not available, we searched the vaccination registration book, which recorded individual-based data at the local commune health center. We also collected administrative coverage data from the Department of Preventive Medicine in Nha Trang on the completed three-primary dose series of DTP vaccine (DTP3) and the fourth dose (DTP4) between 2011 to 2017 in the two study communes (Data at the Department of Preventive Medicine, Nha Trang). Local administrative coverage data before 2011 were not accessible. Thus we collected national administrative coverage data between 1983 and 2017 in Vietnam, obtained from the WHO data repository (WHO 2021).

Serological assay

Collected sera were stored at -80°C in the Pasteur Institute in Nha Trang until testing. Serum anti-diphtheria toxoid IgG level was measured using a commercially available ELISA kit (IBL, Germany) following the manufacturer's protocol. An IgG level of > 0.1 IU/ml in ELISA, the international standard cut-off value for the requirement of a booster dose, was considered seropositive (European Center for Disease Prevention and Control 2014, von Hunolstein. C et al. 2014). The seroprevalence was defined as the proportion of seropositive samples in the total number of samples assessed. We excluded the samples that had errors in sample processing or testing.

Statistical Analyses

Age-stratified seroprevalence and geometric mean concentration (GMC)

Age-stratified seroprevalence and GMC were summarized with 95% confidence intervals using the cross-sectional survey data collected in 2017. To do this, seroprevalence and GMC were weighted by population size in the ten age-sex strata in each commune. The sampling weights were the inverse probability of sampling in that age-sex stratum. The difference in weighted seroprevalence and weighted GMC between the sexes was examined by logistic regression and linear regression, respectively, by overall age and each age stratum. Because an initial inspection of data revealed a large difference in immunity between two younger age groups, the seroprevalence and GMC were calculated by each year of age.

Change in anti-diphtheria toxoid IgG antibody levels

The change in anti-diphtheria toxoid IgG level was examined in participants who participated in both the 2017 (IgG1) and 2019

(IgG2) surveys. Owing to the introduction of the fourth dose of the DTP vaccine in the national immunization program in 2012, many participants who were born after 2011, aged six or younger in 2017, had received four doses after this change. On the contrary, participants aged seven years or older in 2017 had received three doses or less. In addition, observation of plotted IgG values showed that the IgG level declined most when participants were of the age six or younger, whereas it often increased if participants were older than ten years. We, therefore, stratified age by three groups at 0-6, 7-10, and 11-55 years old for our analysis. We also categorized IgG1 into four groups, >0.1, 0.05-0.1, 0.03-0.05, and ≤0.03 IU/ml determined by observation of plotted IgG values, to quantify the change in IgG by IgG1 level.

Change in IgG level over two years was evaluated using a change in natural log-transform IgG values (log(IgG2) – log(IgG1)). IgG levels were logarithmically transformed in statistical analyses to reduce the skewness of the data. Back-transformation of log-transformed changes gives ratios of geometric means. We, therefore, defined the percentage decrease of IgG as (1 - geometric mean of (IgG2/IgG1)) x 100 percent.

The percentage decrease in geometric mean levels over two years was reported for overall and exposure age groups 0-6, 7-10, and 11-55 years, and for IgG1 levels >0.1, 0.05-0.1, 0.03-0.05, and ≤0.03 IU/ml. The percentage decrease in geometric mean levels among age group 0-6 years was reported according to whether three or four DTP vaccine doses were received before the measurement of IgG1 levels. The number of doses received that were confirmed on written vaccination records was used for stratification. The paired t-test was conducted to examine whether the change in natural log-transformed IgG in each group was statistically significant.

Duration of vaccine-derived protective immunity

The duration of vaccine-derived protective immunity was defined as the time since the last vaccination that IgG level was maintained at >0.1 IU/ml. All available paired log-transformed IgG values (log(IgG1) and log(IgG2)) for children aged six or younger in 2017, who were born after 2011, with a history of three or four doses of DTP vaccine before the measurement of IgG1 levels were included in the analysis. Their vaccination histories were confirmed through written vaccination records. We assumed that the immunity of children aged six or younger was attributable to vaccination.

A mixed-effect linear regression model with random intercept incorporating the between-individual variability was used to analyze antibody decay over time for anti-diphtheria toxoid IgG, whose decay pattern was assumed exponential, as reported in previously published studies (Laird and Ware 1982, Renard et al. 2001, Bates et al. 2015, Hammarlund et al. 2016, Antia et al. 2018). The time at which the geometric mean level of IgG declined to 0.1 IU/ml was estimated from the line of best fit. This predicted time can be considered to correspond to the duration of protective immunity. A 95% confidence interval around this duration of protective immunity was calculated by the Delta method (Oehlert 1992, Rice 1994).

Analyses were conducted in the STATA 15 software (StataCorp 2017). R software was used for data visualization (R Core Team (2020)).

Results

A total of 510 participants aged 0-55 years were recruited to the survey in 2017; 221 participants (43%) were male. A total of 306 participants (61%) were followed up in 2019. A written vaccination record was found for 108 participants aged 0-28 years. Ninety-four percent of the participants aged 0-5 years had written vaccination records, and 71% of the participants had received four doses. The vaccination records of 60% of the participants aged 6-15 years were confirmed, and 54% of the participants had received three doses (Table 1).

Vaccination coverage of DTP3 among participants who were born between 2007 and 2017 (aged 0-10 in 2017) in each year varied between 78% and 100% on the basis of oral information and 33% to 100% on the basis of vaccination records. The local and national administrative DTP3 coverage in the same period was between 92% and 100%, except 54% for the year 2013 when the Pentavalent vaccine was suspended in Vietnam during the investigation of one severe adverse event (WHO 2021).

A total of 510 samples collected in 2017 were included to measure seroprevalence for the five age strata. Overall weighted seroprevalence was 26% (95%CI:20%-32%). In this population, the highest seroprevalence was 68% (95%CI:67%-69%) in age group 0-5 years, and the lowest was 7% (95%CI:4%-11%) in age group 6-15 years. Seroprevalence levels in age groups 16-25, 26-35, 36-55 years were 12% (95%CI:7%-19%), 33% (95%CI:27%-40%), and 28% (95%CI:17%-43%), respectively. There was no statistical difference by sex in overall and age-stratified seroprevalence (Table 2).

Seroprevalence constantly decreased from 85% in participants aged one year to 0% in participants aged nine years. It stayed at 0% until the age of twelve and gradually increased afterward (Figure 1A). The highest GMC was 0.75 IU/ml at age one year, and it declined to the lowest GMC of 0.02 IU/ml at age ten years. The decreasing and increasing trend of GMC over age was similar to that of seroprevalence (Figure 1B).

Among 306 samples collected during the follow-up survey in 2019, two samples had errors in sample processing or testing. Therefore, 304 paired samples were available to examine the change in IgG level between 2017 and 2019. Of 304 participants, 50 had a record of three doses, and 49 had received four doses. None of these individuals received DTP vaccine or was diagnosed with diphtheria between 2017 and 2019.

All paired IgG values were plotted by age and number of DTP doses (Figure 2A).The IgG levels declined rapidly in two years among children aged six or younger. Although no consistent pattern of IgG change was observed in children aged seven or older, there was a particular pattern of IgG changes by IgG1 level (Figure 2B).

The percentage decrease of IgG in two years was calculated in different age groups by number of DTP doses and IgG1 levels. The percentage decrease of IgG was 41% (95%CI:28%,52%) over two years among those aged six or younger. IgG levels did not decline significantly among those who were older than six. The percentage decrease constantly diminished in accordance with the decline in IgG1 level. Among those who were younger than seven years, the percentage decreases of IgG after receiving three and four doses of DTP were 34% (95%CI:-8%,59%) and 47% (95%CI:30%,59%), respectively (Figure 2B and Table 3).

The length of time that the geometric mean of IgG was maintained > 0.1 IU/ml was estimated by a mixed-effect linear regression with a random intercept model. The number of samples included in the analysis was 114 (57 individuals) overall, 22 (11 individuals) of whom received three doses only, and 92 (46 individuals) of whom received four doses. IgG was estimated to be maintained at >0.1 IU/ml for 4.7 years (95%CI:3.67-5.63) when the analysis was conducted combining those who received three or four doses (Figure 3). IgG was estimated to be maintained at >0.1IU/ml for 4.3 years (95%CI:3.46-5.26) after receiving four doses. Because of the limited number of participants who received only three doses, we could not estimate the duration of protective immunity among them accurately.

Table 1
The number of study participants by age group in 2017 and follow-up rate and vaccination history by participants' age group in 2019.
Vaccine record for more than 90% of the participants aged 0-5 were available; 71% received four doses. Sixty percent of participants aged 6-15 years had vaccine records, and 54% had a record of receiving three doses.

		Number of participants				F/U rate	Vaccination history among 306 participants in 2019											
year of birth	age (2017)	in 2017 n	(%)	in 2019 n	(%)		with record	(%)	number of vaccines received 4	(%)	3	(%)	2	(%)	1	(%)	0	(%)	no record	(%)
2012-2017	0-5yr	100	20%	63	21%	63%	59	94%	45	71%	9	14%	2	3%	1	2%	2	3%	4	6%
2002-2011	6-15yr	107	21%	65	21%	61%	39	60%	3	5%	35	54%	0	0%	1	2%	0	0%	26	40%
1992-2001	16-25yr	105	21%	52	17%	50%	7	13%	0	0%	6	12%	0	0%	0	0%	1	2%	45	87%
1982-1991	26-35yr	94	18%	59	19%	63%	3	5%	1	2%	1	2%	0	0%	0	0%	1	2%	56	95%
1962-1981	36-55yr	104	20%	67	22%	64%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	67	100%
	All	510	100%	306	100%	60%	108	35%	49	16%	51	17%	2	1%	2	1%	4	1%	198	65%

Ninety-four percent of the participants aged 0-5 years had a written vaccination record; 71% received four doses. A total of 60% of the participants aged 6-15 years had vaccine records, and 54% had a record of three doses. Difference in vaccine history of DTP3 and DTP4 in the youngest two age groups occurred because the national vaccination program introduced the fourth dose in 2012. Participants aged >36 years in 2017 were not vaccinated because DTP was not introduced in Vietnam until 1981.

Table 2
Seroprevalence (seropositive was defined as IgG value >0.1 IU/ml) and the geometric mean concentration (GMC) weighted by population size, age, and sex structures in two communes in Nha Trang city in 2017.

All		Seroprevalence					GMC		
age group	mean age, ±SD(year)	N	n	%	95% CI	*p-value*(Male vs Female)	IU/ml	95% CI	*p-value*(Male vs Female)
0-5yr	3.8 ±0.02	100	68	68%	(67%, 69%)		0.22	(0.21, 0.22)	
6-15yr	11.1 ±2.74	107	7	7%	(4%, 11%)		0.03	(0.02, 0.04)	
16-25yr	20.2 ±5.92	105	13	12%	(7%, 19%)		0.04	(0.03, 0.05)	
26-35yr	31.1 ±1.19	94	30	33%	(27%, 40%)		0.07	(0.06, 0.08)	
36-55yr	45.7 ±2.44	104	30	28%	(17%, 43%)		0.07	(0.04, 0.11)	
total	28.9 ±3.01	510	148	26%	(20%, 32%)		0.06	(0.05, 0.07)	
Male									
0-5yr	3.8 ±0.37	50	32	64%	(60%, 68%)		0.22	(0.15, 0.32)	
6-15yr	11.1 ±5.5	60	3	5%	(3%, 8%)		0.03	(0.02, 0.04)	
16-25yr	20 ±1.97	46	4	8%	(0%, 66%)		0.03	(0.02, 0.06)	
26-35yr	30.8 ±0.46	30	12	39%	(24%, 57%)		0.08	(0.06, 0.09)	
36-55yr	45.1 ±4.88	35	9	26%	(23%, 29%)		0.07	(0.05, 0.09)	
total	28.2 ±4.76	221	60	25%	(19%, 31%)		0.06	(0.05, 0.07)	
Female									
0-5yr	3.9 ±0.45	50	36	72%	(69%, 75%)	0.20	0.21	(0.14, 0.33)	0.90
6-15yr	11.1 ±1.73	47	4	9%	(3%, 24%)	0.52	0.03	(0.03, 0.04)	0.21
16-25yr	20.4 ±6.57	59	9	16%	(8%, 28%)	0.69	0.04	(0.04, 0.04)	0.50
26-35yr	31.4 ±1.45	64	18	28%	(27%, 29%)	0.35	0.06	(0.06, 0.06)	0.28
36-55yr	46.2 ±2.71	69	21	30%	(12%, 57%)	0.71	0.07	(0.04, 0.13)	0.58
total	29.7 ±2.34	289	88	27%	(18%, 37%)	0.66	0.06	(0.05, 0.08)	0.39

Sex difference in seroprevalence was compared using logistic regression and sex difference in GMC was compared using linear regression.

Table 3
The percent reduction of IgG among four baseline IgG levels, five age groups, and number of doses of DTP.

		Percentage decrease of IgG in two years			paired t-test
		N	% decrease	95%CI	*p-value*
all paired samples		304	16%	(8%,24%)	<0.01
age groups	0-6 years	66	41%	(28%,52%)	<0.01
	7-10 years	27	-18%	(-40%,1%)	0.06
	11-55 years	211	11%	(0%,20%)	0.09
baseline IgG level in 2017	>0.1IU/ml	90	56%	(48%,63%)	<0.01
	0.05-0.1IU/ml	73	15%	(5%,24%)	<0.01
	0.03-0.05IU/ml	61	2%	(-12%,15%)	0.72
	<=0.03IU/ml	80	-50%	(-77%,-28%)	<0.01
age 0-6 years only	3 or 4 doses	57	44%	(30%,55%)	<0.01
	3 doses	11	34%	(-8%,59%)	0.65
	4 doses	46	47%	(31%,59%)	<0.01

Percentage decrease = (1- geometric mean of (IgG2/IgG1) *100 percent IgG1: IgG measured in 2017, IgG2: IgG measured in 2019

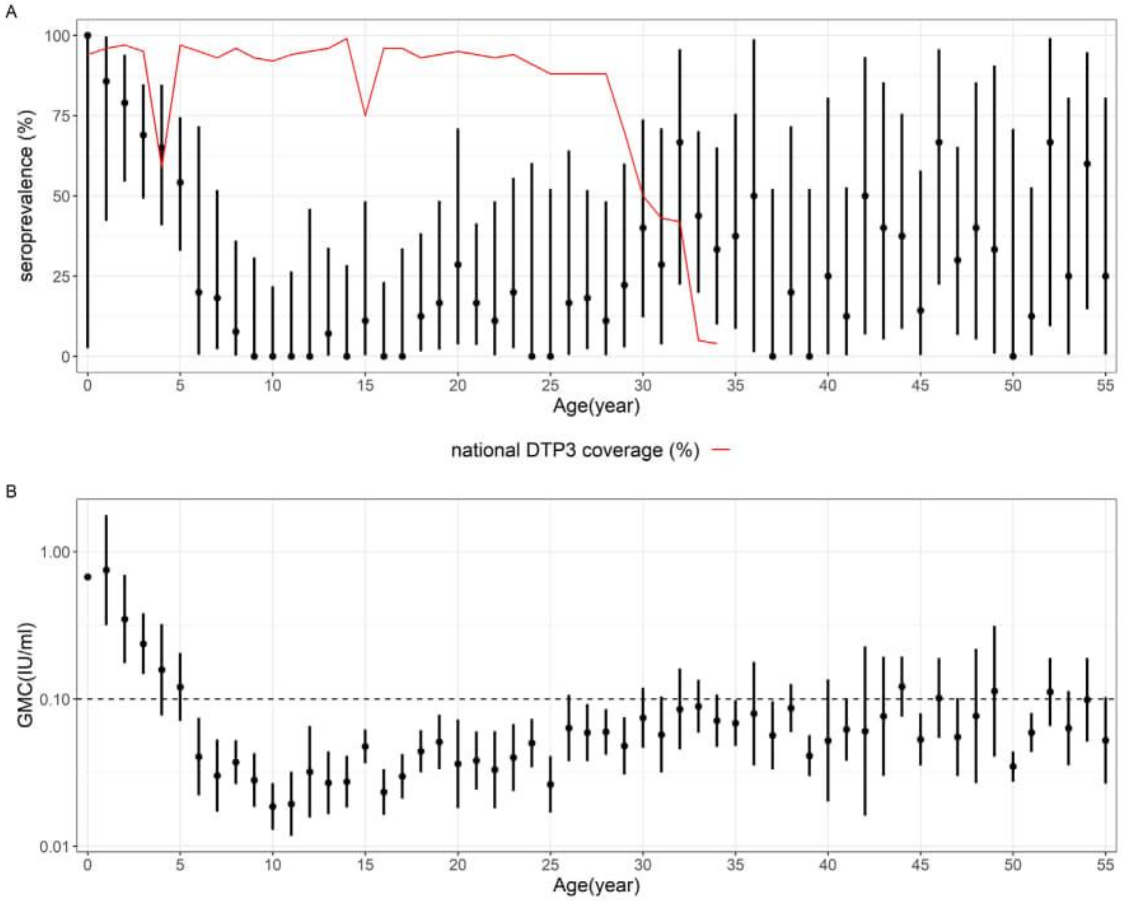

Figure 1. A. Seroprevalence with 95% CI and nationwide DTP3 coverage by single age strata. The low DTP3 coverage in those aged four and 15 years reflects the nationwide suspension of Pentavalent vaccines in 2013 and the stock out of DTP vaccines in 2017.
B. GMC with 95% CI. Dotted horizontal line represents the cut-off for protective immunity (0.1IU/ml).

Discussion

This study describes the age-stratified seroprevalence from infants to adults in well-vaccinated communities where no school-entry booster dose has been introduced. The study shows that 30 years of an immunization program, which provided three or four doses of DTP vaccines within the first two years of life, created a large immunity gap in the study population. Although the reported coverage was high, the seroprevalence was generally low among those aged 6-55 years. The age group in which the reported cases most frequently fell (6-15 years) and the broad age range of cases (1-55 years) in the last few years in Vietnam were consistent with observed seroprevalence pattern by age. The rapid waning of immunity against diphtheria after vaccination may explain the increase in diphtheria cases in Vietnam and other countries in the late 2010s (Wanlapakorn et al. 2014, Hughes et al. 2015, Sein et al. 2016, Sangal et al. 2017, WHO 2017).

Seroprevalence declined quickly from age one to nine years. This result differs from the report that showed that after three doses of DTP vaccine in Germany, antibodies did not wane for the first ten years (Hasselhorn et al. 1998). On the contrary, the pattern of declining seroprevalence in Sweden is similar to that observed in Vietnam (Edmunds et al. 2000). The difference in seroprevalence between populations may be due to the different vaccine components, schedule, and coverage, or the degree of natural infection, but none of them explain the difference clearly. Seroprevalence and GMC tended to increase after ten years of age, which suggests either ongoing transmission of toxigenic *C.diphtheriae* or residual immunity from the transmission in the past.

The seroprevalence among children younger than five years was reported to be 64% in Lao PDR in 2012, which was similar to ours (Nanthavong et al. 2015). The seroprevalence between age 15 and 24 years was 43% in another study in Lao in 2013, and that between age 6 and 25 years was 32% and 52%, respectively, in Kon Tum province in Vietnam in 2016 (Black et al. 2015, Le et al. 2017). The seroprevalence among individuals aged 6-15 and 16-25 years in Lao and Kon Tum was higher than our results, which might reflect natural infection of toxigenic *C. diphtheriae* because both study areas reported cases during the study period.

Although Nha Trang has had no reported diphtheria cases for at least the last ten years, seroprevalence among participants aged 0-55 years was as low as 26% (95%CI:20-32%). The absence of cases in Nha Trang may be attributable to the high DTP3 coverage among infants. Diphtheria cases have been reported where the infant DTP3 coverage was low, e.g., 23% in Rohingya refugee camps in 2017 (Rahman and Islam 2019) and 43% in Nigeria in 2011 (Besa et al. 2014). High immunity among young children may protect the community from large outbreaks although the immunity among older children and adults is low. There are several other potential explanations for the absence of cases despite the low seroprevalence. Our result might not accurately measure the protection level in individuals because we did not use a neutralization assay,

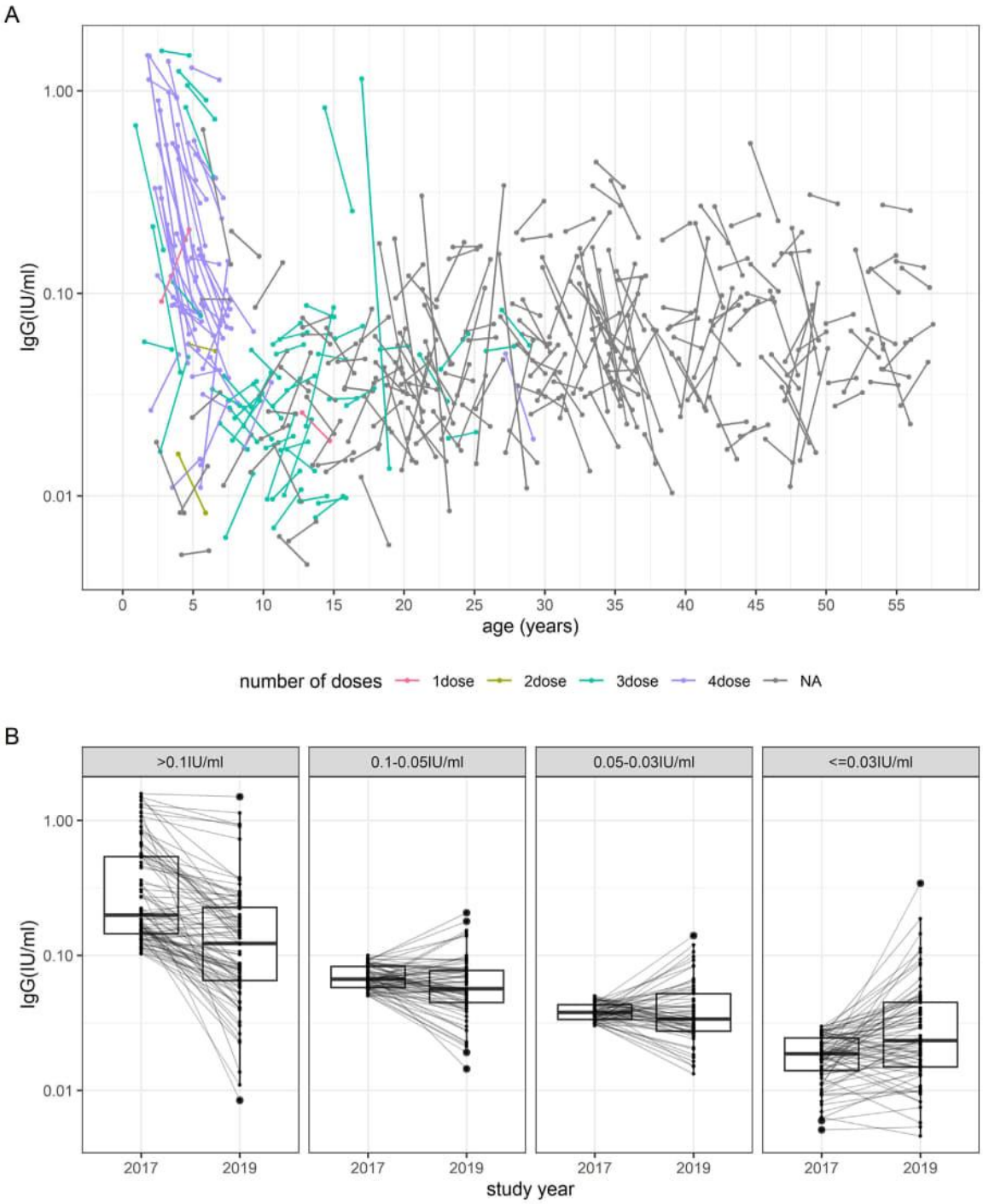

Figure 2. Change of antibody (IgG) levels in 304 paired sera between 2017 and 2019 plotted over age by different number of DTP doses (A) and plotted by four categories of baseline IgG levels with boxplot showing median and first and third quantile of IgG in 2017 and IgG in 2019 (B).

a gold-standard assay. Cellular immunity may persist after vaccination and protect individuals although antibodies are not detected (Gunatillake and Taylor 1968, Heyworth and Ropp 1973).

According to historical data in North America before the introduction of the vaccine, clinical diphtheria was more common in boys among children and in women among adults (Crum 1917). After vaccination was introduced, women were likely to have lower antibody levels than men (Edmunds et al. 2000, Galazka 2000, Plotkin et al. 2018). Biological differences, social role of women, or frequent opportunities of vaccination for men because of military services were discussed. But no clear reasons were identified. This study examined the sex difference in seroprevalence but found no significant difference.

This study shows no evidence of any difference in the waning rate of immunity between receiving three doses or four doses of DTP, although this may be because of the small sample size. The antibody level waned rapidly among individuals with high antibody levels and the antibody level increased among individuals with low antibody levels. This finding suggests that vaccine-derived immunity against diphtheria toxoid is not maintained for life. After it has waned, immunity may increase with subsequent subclinical or clinical infection, as previously reported

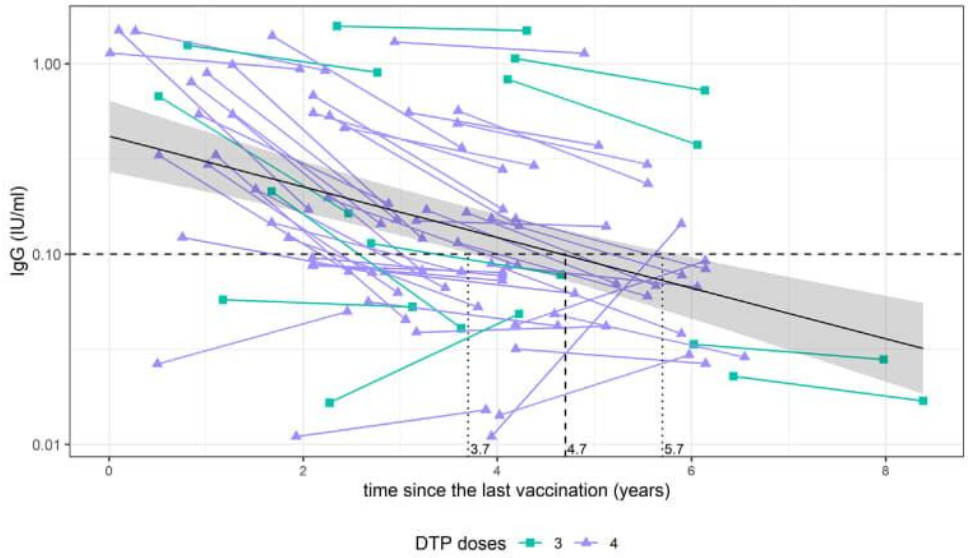

Figure 3. Predicted geometric mean of IgG since the last vaccination after three or four doses of DTP with 95% confidence interval.
Black line and gray area: Geometric mean of IgG concentration since the last vaccination and 95% confidence interval estimated by a mixed effect random intercept model.
Green squares and purple triangles: IgG concentration of each individual who received three doses and four doses of DTP, respectively.
Dotted line shows the protective threshold 0.1 IU/ml. After three or four doses of DTP were given, predicted log-transformed anti-diphtheria toxoid IgG declined linearly and crossed 0.1 IU/ml at 4.7 years after the last vaccination.

(Burnet 1972). On the contrary, regression to the mean could also explain that a high antibody level is likely to decline and a low level of antibody likely to increase (Barnett et al. 2004).

In this study, IgG was estimated to be maintained at a concentration of more than 0.1 IU/ml for 4.3 (95%CI:3.5-5.3) years after four doses of vaccine. Given that the fourth dose is currently scheduled at 18 months of age, 95% of children lose protection between ages of 5.0 and 6.8 years, and thus a booster dose should be administered at school entry, which is at age six in Vietnam. Because DTP vaccine was replaced in Vietnam by Pentavalent vaccine in 2011 and a booster dose at 18 months was introduced in 2012, most of the participants in this analysis had received vaccines according to the current vaccination program. Therefore, the recommendation drawn from this study results will be suitable for Vietnam. This is also compatible with the current WHO recommendation.

This study has several strengths. We used written records to confirm the participants' last vaccination date, which provided accurate vaccination history and time since the last vaccination in each individual. We used paired sera collected in the longitudinal study to estimate the waning of immunity over time.

There are several limitations to this study. It was conducted with a small sample size in a limited geographical area. We collected data only at two points at a fixed interval. The follow-up duration was short, and the results might be affected by random error as there were only two data points. The vaccination history could not be confirmed among older participants, and thus the long-term effect of vaccine-derived immunity could not be evaluated.

An exponential decay model was used to estimate the length of time of vaccine protection up to seven years of age in this study. On the contrary, a previous study used a power function decay model to analyze the waning of anti-diphtheria toxoid IgG (Swart et al. 2016). Another longitudinal study following up the same individuals for seven years showed a biphasic waning pattern of anti-diphtheria toxoid IgG (Nakayama et al. 2019). The exponential decay model may not best fit, especially when follow-up time is longer.

Conclusions

This study showed that the population aged six years or older was largely susceptible in the study community in Vietnam, where the DTP3 coverage was high in the last ten years except in 2013. If any cases were imported or DTP3 coverage declined for some reason, there is a potential risk of re-emergence of the disease. Considering these risks, a cost-effectiveness analysis for introducing an additional booster dose may be warranted. The high case fatality ratio of diphtheria would justify a booster dose at school entry to reduce preventable deaths. The study also showed that a school-entry booster dose would be required to maintain immunity against diphtheria in Vietnam.

Conflict of Interest

There is no conflict of interest to be declared.

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Ethical consideration

Ethical approval was obtained from the Vietnamese Ministry of Health and the London School of Hygiene and Tropical Medicine ethical review boards (IRB-VN01057-27/2015, LSHTM Ethics ref: 17518/17913).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2022.01.025.

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Chapter 6: Seroepidemiology and carriage of diphtheria in an epidemic-prone area and implications for vaccination policy in Vietnam

Chapter overview

This chapter aims to elucidate the mechanism of a recent diphtheria outbreak in Vietnam and to discuss an effective response strategy and future vaccination policy based on the seroepidemiology and microbiology of diphtheria in an epidemic-prone area.

Chapter 5 described very low seroprevalence (anti-diphtheria toxoid IgG ≥ 0.1 IU/ml via ELISA) in a well-vaccinated community in Vietnam especially in school-age children. Chapter 6 aims to measure the seroprevalence in an epidemic-prone area where cases continuously occurred in the last decade. The seroprevalence in an epidemic-prone area and in a well-vaccinated community (Chapter 5) are compared.

In Chapter 3, it was suggested that asymptomatic carriers of the non-toxigenic strain can transmit *C. diphtheriae* and contribute to the continuous outbreaks in Vietnam. Asymptomatic carriers play an important role in the transmission of diphtheria; however, this stage of the disease is not well understood. Therefore, the carriage prevalence of healthy hosts who carry toxigenic or non-toxigenic strains was measured in the epidemic-prone area. The risk factors for being a carrier were also examined.

In Chapter 3, it was suspected that vaccination might not be effective in an epidemic-prone area. Therefore, Chapter 6 assesses the association between antibody titre and nutrition as a potential factor for interfering in the immune-response in hosts.

Chapter summary

A cross-sectional community-based carriage prevalence and seroprevalence survey was conducted in Quang Ngai province in Vietnam, an epidemic-prone area where diphtheria cases have been continuously reported for the last 10 years. A total of 1,216 subjects aged 1–55 years were recruited. The study confirmed that 1.4% of the population were asymptomatic carriers of *C. diphtheriae* and that the carriage prevalence declined with age. *C. diphtheriae* was isolated by culture from 17 out of 27 qPCR positive samples: 9 (33%) out of the 27 carriers were positive for the *tox* gene via qPCR, but only 6 of these were successfully recovered by isolation. From those six, diphtheria toxin expression was confirmed in three isolates using the modified Elek test. The remaining three isolates were NTTB strains. We identified 27 carriers who were concentrated in specific households and geographical areas.

The risk factors for being a carrier were examined by logistic regression analysis. Young age was associated with carrier status; however, there was no evidence of association with other

previously reported risk factors, including sex, vaccination history, school attendance, dormitory stay, bed sharing, skin hygiene practice, and nutrition status measured by mid upper arm circumference (MUAC).

Age-stratified seroprevalence in the epidemic-prone area was 40% (95% CI: 23–59), 37% (95% CI: 29–45), 55% (95% CI: 43–67), and 63% (95% CI: 61–65) for 1–5 years, 6–17 years, 18–40 years and 41–55 years, respectively. The seroprevalence among school-age children was the lowest and increased with age; however, observed immunity patterns were significantly different from that in a well-vaccinated community. This suggested that low immunity among children, due to low vaccination coverage, predisposed to transmission resulting in symptomatic and asymptomatic infections in school-age children, in whom vaccine-induced immunity had waned. Therefore, introduction of a school-entry booster dose and high infant DTP coverage are necessary to stop current transmission.

Furthermore, the immunity patterns in the epidemic-prone area suggested that an SIA would be most effective if it targeted the population aged 1–17 years due to their lower seroprevalence, while SIAs targeting 18–40-year-old adults would also be beneficial, as one-half of this age group was susceptible.

The association between anti-diphtheria toxoid antibody levels and nutrition status among children measured by MUAC was examined via multivariate linear regression analysis adjusting for age. Poor nutrition status was associated with lower antibody levels, although nutrition was not associated with carrier status. Considering the high wasting and stunting rate in the area, the low immunity level among vaccinated children may be attributable to malnutrition.

These findings aid in our understanding of the mechanism of diphtheria outbreaks and in identifying the appropriate public health response to control diphtheria in many other LMICs.

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SECTION A – Student Details

Student ID Number	lsh422077	**Title**	Dr.
First Name(s)	Noriko		
Surname/Family Name	Kitamura		
Thesis Title	Understanding factors contributing to outbreaks of diphtheria and implications for vaccination policy in Viet Nam		
Primary Supervisor	Paul Fine		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Emerging Infectious Diseases
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I have planned and designed a research and conducted a data collection an analyses with other co-authors.

SECTION E

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Date	1 Sep 2022

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Date	21 September 2022

Background:

Diphtheria is an infectious disease caused by toxigenic strains of *Corynebacterium diphtheriae, C. ulcerans*, and rarely *C. pseudotuberculosis* (172, 204, 205). While the diphtheria toxoid vaccine contributed to a decrease in the number of diphtheria cases globally, the disease remains a threat to public health, especially in South and Southeast Asia (206, 207). Currently, the World Health Organization (WHO) recommends three primary doses of the diphtheria-tetanus-pertussis (DTP) vaccine in early infants (i.e., at 6, 10, and 14 weeks), followed by three booster doses at 12–23 months, 4–7 years and 9–15 years, to protect all age groups. However, many LMICs have not introduced all booster doses.

The Vietnamese Ministry of Health (MoH) first introduced the DTP vaccine in 1981, targeting the 2, 3, and 4 month age groups. A booster dose targeting 18-month-old was introduced in 2011 (124). Due to efforts in vaccination, reported diphtheria cases in Vietnam declined to nearly zero by 2010; however, several small diphtheria outbreaks in remote districts in Central and Western Vietnam have been reported since 2013 (19).

Supplemental immunisation activities (SIAs), in which vaccination is delivered to all targeted individuals regardless of their prior vaccination history, were conducted in the areas surrounding Quang Ngai province when diphtheria cases were identified between 2013 and 2019 (208). However, the majority of the population of Quang Ngai province had not been covered by SIAs as of October 2019. According to the national surveillance programme, Quang Ngai province reported two laboratory-confirmed cases in 2017–2018 and 47 in 2019–2020, among an estimated population of 1,231,697 people (209). Among the cases, 36 (73%) were school-age children (6–17 years old). Among the confirmed cases, three (6%) were fatal.

Although national administrative DTP coverage has been maintained above 90% in Vietnam since 1994 (excluding 2002 and 2013), subnational coverage has not always been high (5). While low vaccination coverage in localised areas appears to cause diphtheria outbreaks, the immune profile of the population in these areas is unknown (206). Currently, the WHO suggests including adults in SIAs to control diphtheria outbreaks, as adults may also be susceptible; however, no specific age groups were recommended because the epidemiological characteristics vary by country (172).

Asymptomatic carriers play an important role in transmission dynamics; however, details of the carrier stage in affected areas are largely unknown because the proportion of healthy carriers who carry toxigenic and non-toxigenic strains has not been investigated in the Southeast Asian region (108, 146). Moreover, host factors that govern carriage status have not been elucidated.

This study primarily aimed to measure the carriage prevalence of *Corynebacterium* species in the respiratory tract in areas where outbreaks occurred and to assess potential risk factors for carriage. The second aim was to measure the age-stratified serological immune profile against diphtheria toxin, which would help to reveal the mechanism of the recent outbreaks and to target the most appropriate age groups of using SIA. Reflecting a previous study suggesting that low antibody levels increased the risk of being a carrier (210), this study also examined the factors that contributed to low immunity among individuals. The third aim was to compare the immune profile patterns in areas where cases have been reported and in areas where cases have not been reported to discuss the current DTP schedule in Vietnam.

Methods:

Study site

Two districts, Tay Tra and Son Ha in Quang Ngai province, were selected as the study area as three diphtheria cases were identified between January and September 2019 and no SIAs had been implemented (Figure 1). Two communes in the Son Ha district were excluded because a mop-up vaccination campaign of DTP was conducted in those communities in 2018. The estimated population of the two districts was 99,121 in 2019 (209). Health access is limited in this study area due to the mountainous topography.

Study design and sampling method

A community-based cross-sectional survey was conducted in October 2019. We stratified the ages into four groups, 1–5 years, 6–17 years, 18–40 years, and 41–55 years, as children go from primary to high school between the age of 6–17 in Vietnam. Based on the previously obtained age-stratified seroprevalence in Vietnam (211, 212), the required sample size for

each age stratum was estimated to be 350, 400, 400, and 350, respectively, with 10% precision, 3.5 design effect, and an 80% response rate.

Multi-stage cluster sampling was conducted. In each district, five communes were sampled by population proportion to size, and three villages were selected from each commune by simple random sampling. In total, 30 villages were selected (Chapter 6 Figure 1). Because the average household size in Vietnam is four members (126), 15 households in each village for, a total of 450 households were selected to recruit 1,500 individuals. Households with children aged 1–5 years were oversampled to recruit a higher proportion of the sample size than the original population. More specifically, Vietnamese census data in 2019 which listed all the households including age of all household members was used for the random sampling of household in the study area. Five households with children aged 1–5 years were randomly selected while 10 households were randomly selected from all households in each commune.

Data and sample collection

Local healthcare workers visited the participants' homes to invite them to take part in the survey. After participants arrived at the survey site, written informed consent was obtained from each participant or guardian. The survey teams interviewed each participant using a standardised questionnaire and collected information on sex, age, vaccination history, and other socio-demographic information. Based on the previously reported risk factors for diphtheria infection or carriage of *C. diphtheriae*, age, vaccination history, seropositivity (anti-diphtheria toxoid IgG ≥ 0.1IU/ml), bed-sharing, school attendance, staying in school dormitories, household size, frequency of bathing or handwashing, having livestock or companion animals, anti-diphtheria toxoid IgG level, and mid-upper arm circumference (MUAC) were assessed for their association with the carriage of *Corynebacterium* species (17, 45-47, 210, 213). MUAC was used as a measure of the nutritional status of children aged 15 years. Vaccination history was collected for children aged 10 years or younger from either the participants' vaccination card or the vaccine registration book from the respective community health centres in their residence area.

Dried blood spot (DBS) were collected by venepuncture or finger prick on a Whatman 903 protein saver card (#Z761575) and stored at -80 °C according to the procedure referenced by the US Centers for Disease Control (214, 215). Throat and nasopharyngeal swabs were

collected and stored in Amies medium and STGG medium, respectively (205). Collected samples were stored at -30 °C at Quang Ngai Provincial Health Service until transported to the Pasteur Institute in Nha Trang, where they were stored at -80 °C until testing. Ethical approval was obtained from the ethical review boards of the Pasteur Institute in Nha Trang, Vietnamese MoH, Nagasaki University, and the London School of Hygiene and Tropical Medicine (1775/IPN-DT, 1046/K2DT-KHCN, Nagasaki University IRB-approval number: 191226228, LSHTM ethics ref:17518).

Microbiological tests

The collected swabs were cultured on Tellurite-containing agar medium in a 35 °C incubator for 2448 hours (205). If black colonies were grown, they were initially tested by Gram stain to identify gram-positive bacilli (205). The species and biovars were identified for each subculture using the API Coryne test (bioMérieux) (205). They were tested for expression of the diphtheria toxin using the modified Elek test (216).

Quadruplex real-time PCR (qPCR) was conducted directly on throat swabs and aliquots of STGG medium to identify *C. diphtheriae, C. ulcerans or C. pseudotuberculosis* and the diphtheria toxin gene following previously published methods (205, 217). DNA was extracted using the QIAmp DNA extraction kit (QIAgen, USA) (218). Primers and probes targeted two *rpoB* genes, the *tox* gene, and the green fluorescent protein gene (*gfp*) for internal positive control.

Anti-diphtheria toxoid serological assay

Anti-diphtheria toxoid IgG level was measured by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Binding Site, UK) following the manufacturer's protocol. A DBS was punched out with a 6-mm hole punch and stored in Eppendorf tubes. The DBS was eluted with 500µl elution buffer and incubated overnight at 4 °C. The supernatant of the eluted solution was collected and used for ELISA (219-222). IgG ≥ 0.1 IU/ml, an international standard cut-off value, was defined as seropositive (223, 224). Seroprevalence was calculated by the number of seropositive samples divided by the total number of participants.

Comparison of seroprevalence in the two areas with or without reported cases

This study compared seroprevalence in an epidemic-prone area (Quang Ngai province) and a non-epidemic area (Nha Trang) in Vietnam. Regarding the non-epidemic area, Nha Trang city in Khanh Hoa province was selected as the population is well-vaccinated and has not reported any diphtheria cases since 2013. Moreover, the age-stratified seroprevalence data among those aged 155 years were investigated in Nha Trang city in 2017 (212). Therefore, we compared the immunity pattern of the population in Quang Ngai province with Nha Trang City.

Two different ELISA kits were used for measuring anti-diphtheria toxoid IgG, Binding Site (UK) for the Quang Ngai's study and IBL (Germany) for the Nha Trang study. First, 546 subsets of the samples collected in Quang Ngai were tested by two ELISA kits in parallel, and the two results were compared by linear regression analysis. Based on the best-fitted line, the log-transformed IgG value measured by Binding Site was converted to the value of IBL by the formula below (Figure 2).

Y(log-IgG-IBL) = -0.7652 + 0.72197X (log-IgG-Binding Site)

Then, seroprevalence in Quang Ngai was recalculated using the converted IgG concentration and stratified into five age groups of 1–5, 6–15, 16–25, 26–35, and 36–55 years. Finally, age-stratified seroprevalence and 95% confidence intervals (CI) in Quang Ngai and Nha Trang were compared.

Statistical analysis

Carriage prevalence and seroprevalence were measured with 95%CI after being weighted by population size. Sampling weight was calculated by the inverse proportion of the sample size to the population in each district and age group. Socio-demographic information on the participants was summarised by district. The differences in characteristics of the two districts were examined by χ^2-test or *t*-test.

Fisher's exact test or t-test was conducted to examine the association between carriage status and each risk factor. Multivariate logistic regression analysis was conducted to confirm whether carriage status was associated with young individuals or low IgG levels. As nutrition is a critical element for immune response, multivariate linear regression analysis was conducted to explore the association between an individual's immunity level (natural log-transformed IgG) and nutrition status (MUAC), with adjustment for age. Statistical analyses were conducted by using STATA 15 software (225).

Results:

A total of 1,216 individuals were recruited from 458 households. 269 (22%), 322 (26%), 523 (43%), and 102 (8%) participants were aged 1–5, 6–17, 18–40, and 41–55 years, respectively, and 615 (51%) were male. In total, 75%, 74%, and 43% of children aged 10 years or younger had received at least one dose, three doses, and four doses of DTP (DTP1, DTP3, and DTP4), respectively. There was no statistically significant difference in DTP3 or DTP4 coverage between the two districts. No participants recalled any symptoms or diagnosis of diphtheria in the past. No participants had received the DTP or tetanus-diphtheria (Td) vaccine due to injuries or involvement in the recent SIAs. Regarding ethnicity, 80% of participants in Tay Tra district were of the *'Co'* ethnic group, and 87% of participants in Son Ha district were of the '*Hre*' ethnic group. Vietnam's major ethnic group, *'Kinh*,*'* accounted for only a small proportion of the population in the two districts. Most of the adult participants (91%) were farmers (Chapter 6 Table 1).

Overall weighted carriage prevalence of *Corynebacterium* species was 1.4% (95% CI:0.4–5.3), and the prevalence of the *tox* gene-bearing strain was 0.5% (95% CI: 0.0–4.7). Age-stratified carriage prevalence levels were 4.5% (95% CI: 3.7–5.5), 2.5% (95% CI: 0.0–47.5), 1.0% (95% CI: 0.6–1.7), and 0.0% (95% CI: NA) for 1–5, 6–17, 18–40 and 41–55-year age groups, respectively. The overall weighted seroprevalence of anti-diphtheria toxoid IgG (≥ 0.1IU/ml) in the study area was 51% (95% CI: 44–59): Age-stratified seroprevalence levels were 40% (95% CI: 23–59), 37% (95% CI: 29–45), 55% (95% CI: 43–67), and 63% (95% CI: 61–65) for 1–5, 6–17, 18–40 and 41–55 years old, respectively (Chapter 6 Table 2).

We identified 27 carriers by qPCR. All of them carried *C. diphtheriae* confirmed by qPCR. Among the identified carriers, 17 (63%) were female, and 10 (37%) were male. Among the 17 females, 10 were 1–5 years old; among the 10 male carriers, 2 were 1–5 years old. Sixteen carriers had received at least three doses of DTP. *C. diphtheriae* was isolated by culture from 17 out of 27 qPCR positive samples; 11 were biovar *mitis,* and 6 were *gravis*. Swabs from 9 of the 27 carriers (33%) were *tox*-gene positive by qPCR; however, only 6 of these were successfully recovered by isolation. From those six, diphtheria toxin expression was confirmed in three isolates using the modified Elek test (two *C. diphtheriae* biovar *mitis*, one biovar *gravis*). The remaining three isolates did not express diphtheria toxin and were thus *tox* gene-bearing non-toxigenic strains (NTTB); all three belonged to the biovar *mitis* (Chapter 6 Table 3).

We identified 27 carriers from 21 households located in 8 communes. Of 27 carriers, 10 lived in a commune called ‘Son Ha commune,’ where 12 additional laboratory-confirmed cases were identified within 1 month of the survey date. Out of 21 households, more than 1 carrier were identified in 5 households; 4 households had 2 carriers, and 1 household had 3 carriers. Sixteen carriers had received at least three doses of DTP (Chapter 6 Table 3).

There was strong evidence that age and IgG level were associated with carriage status (Table 4). Young children were likely to be carriers after adjusting for IgG level. High IgG level was unexpectedly associated with carriers after adjusting for age. Multivariate linear regression analysis showed that smaller MUAC was associated with low IgG level after adjusting for age; however, MUAC was not associated with carriage status (Chapter 6 Tables 4 and 5).

The overall seroprevalence was significantly higher in Quang Ngai (52% [95% CI: 49–55]) than Nha Trang (26% [95% CI: 22–30]). The seroprevalence among children aged 1–5 years was lower in Quang Ngai (36% [95% CI :31–42]) than in Nha Trang (68% [95% CI :52–81]), and the seroprevalence among 6–15-year-old children in Quang Ngai (34% [95% CI :29–40]) was significantly higher than in Nha Trang (7% [95% CI :2–14]) (Chapter 6 Figure 3).

Discussion:

We conducted this study to investigate the potential mechanisms underlying the recent outbreaks of diphtheria in Vietnam and to recommend a reasonable outbreak response and vaccination strategy. This study described the community-based *C. diphtheriae* carriage prevalence in a diphtheria epidemic-prone area and assessed potential risk factors for carrier status and low immunity among individuals. Furthermore, we highlighted the difference in population immunity between the epidemic-prone and non-epidemic areas.

The carriage prevalence, especially the prevalence of toxigenic strains, in the study population was much higher than the recently reported prevalence in Europe. According to a European multi-country study conducted in 2007–2008, the prevalence of toxigenic strains in eight European countries was 0% (122). Toxigenic strains were isolated only in Latvia

(0.08%) and Lithuania (0.07%), with over 1,500 cases and 112 cases reported since 1994 (102, 123). The prevalence of non-toxigenic strains was reported to be 0.4% in Turkey in the same study. (122) In our study, carriage prevalence was highest in the youngest age group and declined with age. In Italy, 0.15% of healthy children aged 6–14 years carried a non-toxigenic strain in the early 2000s (226), while in Indonesia, the prevalence of toxigenic strains was reported to be 3% among 1–15-year-old children during the outbreak in 2012 (118). Therefore, the long-running child vaccination programme in Europe appears to have reduced carriage prevalence, especially the carriage prevalence of toxigenic strains. However, toxigenic strains were still identified in countries where symptomatic cases were reported in the last 10 years. In addition, the current carriage prevalence in Vietnam was similar to the situation in the UK in 1971 (1.2%) (121). Considering that the DTP vaccine was introduced in the UK in 1941, 40 years earlier than in Vietnam, vaccination coverage should become adequate in the next few decades to reduce the carriage prevalence of toxigenic strains in Vietnam. The high prevalence of toxigenic strain indicates that more cases may be observed if the population remains susceptible.

Nine out of twenty-seven carriers (33%) harboured *tox* gene-bearing strains. The remaining 18 carriers harboured non-toxigenic strains, which rarely cause invasive diseases (227, 228). On the other hand, non-toxigenic strains often play an important role in maintaining the transmission of *C. diphtheriae* among human hosts (108, 152). Non-toxigenic strains can be converted to toxigenic strains by lysogenisation with a specific temperate bacteriophage. Lysogenic conversions may occur in non-toxigenic strains in carriers, and the converted strains may infect others (138). Multi-locus sequence typing of the identified strains from carriers and cases in this study may provide evidence to indicate that this conversion might have occurred in this community.

Regarding nine *tox* gene-bearing strains, all three healthy carriers with the NTTB strain had received three doses of DTP, which supports the observation that NTTB strains are increasingly identified in Europe due to vaccine pressure (107, 146). The current vaccine does not protect individuals from NTTB strains (103). Although it is unlikely that NTTB strains will be an immediate threat in Vietnam, it may be necessary to monitor NTTB strains as a potential cause of disease in the future.

We found that carriers were concentrated in specific households and communities. This observation was consistent with household transmission being the main route of *C. diphtheriae* transmission in the pre-vaccination era (154). Once diphtheria appears in a household or specific community, transmissions may continue if the neighbouring areas are not sufficiently vaccinated (138).

We found no association between carrier status and bed-sharing, staying at the school dormitory, or less frequent bathing, while several other studies identified them as risk factors for infection (17, 45-47, 213). The number of carriers was so small that there was no adequate power to assess these risk factors. In addition, biological characteristics, such as age or individual immunity level, might have been more important than social factors. At an aggregated level, carriage prevalence was negatively associated with seroprevalence against diphtheria. However, we could not identify an association between carrier status and low IgG level at the individual level. The result of the logistic regression shows that if individual's IgG level gets higher by 1.0 IU/ml, the individuals are 1.41 times higher chance to be a carrier of *C. diphtheriae*. This is probably because of the natural boosting of immunity after being a carrier. As this study was cross-sectional, we could not directly prove the chronological change in an individual's immunity and carriage status.

We confirmed that the lowest seroprevalence (37%) was in 6–17-year-old children, as was expected from the previous finding that most of the laboratory-confirmed cases were school-age children (19). In addition, the seroprevalence was similarly low (40%) among children aged 1–5 years, which may due to low DTP3 coverage and the waning of vaccine-derived immunity. Another potential reason is that the seroconversion rate after DTP vaccination might have been low due to host factors, such as malnutrition, or external factors, such as suboptimal cold chains. In Quang Ngai province, wasting as reported in 5.7% of children under the age of 5 years, and 25.5% were reported to be stunted in 2013 (229). As small MUAC was associated with low anti-diphtheria toxoid IgG, poor nutrition status may be associated with low immune response in individuals.

The age-stratified seroprevalence in Quang Ngai province compared with Nha Trang city provided insights into waning and acquired immunity. The seroprevalence among children aged 1–5 years in Quang Ngai was significantly lower than in Nha Trang, most likely due to the low vaccination coverage in Quang Ngai. In contrast, the seroprevalence among children

aged 6–15 years in Quang Ngai was significantly higher than in Nha Trang, reflecting the continuous natural exposure in Quang Ngai. This observation indicates that the low immunity among children aged 1–5 years led to ongoing transmission, resulting in high seroprevalence among those aged 6–15 years or older in Quang Ngai than in Nha Trang. The same observation was reported in an Indonesian seroprevalence survey in 2012 (118).

The vaccination policy in Vietnam can be discussed from the results. The study population was probably continuously exposed to the pathogen, and the highest number of symptomatic cases was observed in children aged 6–17 years, when vaccine-induced immunity declines most. The population older than 17 years was more protected than younger age groups, probably due to naturally acquired immunity. Nevertheless, one-half of the population over 17 years old was susceptible, which explains why all age groups have been affected by diphtheria recently (206). A school-entry booster dose is recommended to prevent future cases because the infant immunisation program appeared to create low immunity in school-age children (212). However, low immunity in pre-school-age children may be another reason for the recent epidemic in Quang Ngai province. Therefore, improving routine infant vaccination coverage will be essential to controlling diphtheria.

Based on the low seroprevalence in the 1–5 year and 6–17 year age groups, SIAs would be most effective if they targeted the population aged 1–17 years. The Vietnamese MoH so far included the population aged 1–40 years as a target of diphtheria SIAs, while SIAs in Indonesia, Bangladesh, and Haiti have targeted children aged 1–14 (230-232). In Vietnam, targeting the population aged 18–40 years would be beneficial, as half of this age group is susceptible; however, we should also be aware that SIAs would not immediately stop transmission once transmission has started in susceptible populations.

Conclusion:

We found that 1.4% of the population in epidemic-prone area were healthy carriers of *C. diphtheriae*. Two-thirds of them harboured a non-toxigenic strain, which could be transmitted among human hosts asymptomatically. A school-entry booster dose and improved infant vaccination coverage are recommended to decrease the current level of *C. diphtheriae* transmission in Vietnam. SIAs targeting the population aged 1–17 years would be an efficient outbreak response.

Chapter 6 Table 1. Socio-demographic characteristics of participants and households in Tay Tra and Son Ha district in the survey in Quang Ngai province, 2019

Individual data		All (n=1,216)		Tay Tra (n=604)		Son Ha (n=612)		*p-value*
		n	(%)	n	(%)	n	(%)	χ^2 test
Age	≤5yr	269	22%	125	21%	144	24%	0.45
	6-17yr	322	26%	171	28%	151	25%	
	18-40yr	523	43%	258	43%	265	43%	
	40-55yr	102	8%	50	8%	52	8%	
Sex	Male	615	51%	309	51%	306	50%	0.69
	Female	601	49%	295	49%	306	50%	
Ethnic Group	Co	487	40%	486	80%	1	0.2%	<0.01
	Hre	531	44%	0	0%	531	87%	
	K'Dong	110	9%	105	17%	5	1%	
	Kinh	79	6%	5	1%	74	12%	
	Others	9	1%	8	1%	1	0.2%	
≥18 years		All (n=625)		Tay Tra (n=308)		Son Ha (n =317)		χ^2 test
Occupation	farmer	569	91%	278	90%	291	92%	0.50
	others	56	9%	30	10%	26	8%	
≤10years		All (n=464)		Tay Tra (n=231)		Son Ha (n=233)		χ^2 test
Confirmed vaccination history	BCG	361	78%	165	71%	196	84%	<0.01
	DTP1	347	75%	165	71%	182	78%	0.12
	DTP3	343	74%	163	71%	180	77%	0.12
	DTP4	198	43%	90	39%	108	46%	0.13
	Measles	350	75%	160	69%	190	82%	0.02
		All (n=235)		Tay Tra (n=111)		Son Ha (n=124)		
		mean	(SD)	mean	(SD)	mean	(SD)	*t*-test
MUAC	(cm)	14.7	1.3	14.6	1.4	14.8	1.2	0.20
Household data		All (n=458)		TayTra (n=252)		Son Ha (n=206)		χ^2 test
		n	(%)	n	(%)	n	(%)	or fisher
Toilet facility +		323	71%	215	85%	108	52%	<0.01
Water source	Well	168	37%	2	1%	166	81%	<0.01
	River	249	54%	247	98%	32	16%	<0.01
Energy source	Gas	95	21%	22	9%	73	35%	<0.01
	Bio fuel	358	78%	226	90%	132	64%	<0.01

Chapter 6 Table 2. Age-stratified carriage prevalence of *C. diphtheriae* and seroprevalence of anti-diphtheria toxoid IgG (≥0.1IU/ml) in two districts in Quang Ngai province, Vietnam

		mean age		**Weighted seroprevalence**			**Weighted carriage prevalence**		
	age group	mean±SD (year)	N	n	%	[95%CI]	n	%	[95%CI]
All	≤ 5 yr	3.2±1.36	269	108	43.4	[34.3 , 52.9]	12	2.7	[0.6 ,7.5]
	6-17 yr	10.1±3.17	332	120	37.1	[31.5 , 43.1]	10	2.6	[0.1 ,5]
	18-40yr	29.5±5.61	513	283	53.5	[48.7 , 58.3]	5	0.8	[0.2 ,2.1]
	41-55yr	46.3±4.30	102	64	63.2	[58.2 , 68.0]	0	0.0	[0.0, 0.9]
	Over all	20.0±14.3	1,216	575	51.9	[49.0 , 54.8]	27	1.1	[0.6 ,1.9]
		mean age		**Seroprevalence**			**Carriage prevalence**		
	age group	mean±SD (year)	N	n	%	[95%CI]	n	%	[95%CI]
Tay Tra district	≤ 5 yr	3.1±1.42	125	41	32.8	[25.1 , 41.5]	6	4.8	[2.2 ,10.3]
	6-17 yr	9.9±3.09	171	59	34.5	[27.8 , 41.9]	0	0.0	[0.0 , 2.1]
	18-40yr	29.7±5.19	258	152	58.9	[52.8 , 64.8]	3	1.2	[0.4 ,3.5]
	41-55yr	46.4±4.69	50	31	62.0	[47.9 , 74.3]	0	0.0	[0.0 , 7.1]
	total	20.0±14.2	604	283	50.6	[33.6 , 67.5]	9	0.9	[0.2 ,3.7]
Son Ha district	≤ 5 yr	3.2±1.31	144	67	46.5	[38.5 , 54.7]	6	4.2	[1.9 ,9]
	6-17 yr	10.4±3.25	151	61	40.4	[32.9 , 48.4]	10	6.6	[3.6 ,11.9]
	18-40yr	29.4±6.04	265	131	49.4	[43.4 , 55.4]	2	0.8	[0.2 ,3]
	41-55yr	46.2±3.94	52	33	63.5	[49.7 , 75.3]	0	0.0	[0.0 , 6.8]
	total	20.0±14.4	612	292	52.4	[39.1 , 65.3]	18	2.0	[0.3 ,11]

Chapter 6 Table 3. Geographical distribution, characteristics, and vaccination history of 27 carriers of *C. diphtheriae* identified during the study

No	District	Commune	Village	HH number	Age	Sex	Species	*tox gene*	Biovar	Elek test	Vaccine status	DTP3 coverage (%)
1	TayTra	Tra Phong	Tra Nga	305	25	M	*C.diph*	-	gravis	-	NA	60[35,81]
2		Tra Thanh	Thon Mon	818	23	M	*C.diph*	-	na	na	NA	76[51,91]
3*		Tra Lanh	Tra Luong	1003	40	F	*C.diph*	-	mitis	-	NA	64[34,86]
4		Tra Xinh	Tra Kem	1401	5	F	*C.diph*	-	na	na	4 doses	85[55,96]
5				1404	2	F	*C.diph*	-	na	na	4 doses	
6				1407	4	F	*C.diph*	-	na	na	4 doses	
7			Tra Veo	1501	3	F	*C.diph*	-	mitis	-	4 doses	63[38,82]
8					5	F	*C.diph*	-	mitis	-	4 doses	
9				1505	2	F	*C.diph*	+	mitis	-	4 doses	
10	Son Ha	Son Ha	Deo Ron	1607	10	F	*C.diph*	+	na	na	0 dose	71[44,89]
11					3	F	*C.diph*	-	mitis	-	3 doses	
12					2	F	*C.diph*	+	na	na	4 doses	
13			Dong Reng	1704	9	M	*C.diph*	-	na	na	0 dose	40[19,65]
14				1707	14	M	*C.diph*	+	mitis	+	NA	
15			Ha Bac	1807	9	M	*C.diph*	+	na	na	0 dose	71[46,87]
16				1811	7	F	*C.diph*	+	mitis	+	4 doses	
17					10	M	*C.diph*	+	mitis	-	3 doses	
18				1812	10	M	*C.diph*	+	mitis	-	3 doses	
19					4	M	*C.diph*	-	mitis	-	0 dose	
20		Son Giang	Lang Ri	2314	10	F	*C.diph*	-	mitis	-	0 dose	94[68,99]
21			Ta Dinh	2406	28	M	*C.diph*	-	gravis	-	NA	93[63,99]
22					25	F	*C.diph*	+	gravis	+	NA	
23				2409	3	M	*C.diph*	-	gravis	-	4 doses	
24		Son Ky	Lang Re	2505	7	F	*C.diph*	-	na	na	4 doses	88[61,97]
25		Son Hai	Lang Trang	3004	7	F	*C.diph*	-	gravis	-	3 doses	80[57,92]
26				3011	2	F	*C.diph*	-	na	na	3 doses	
27				3012	5	F	*C.diph*	-	gravis	-	3 doses	

* No.3: *C. diphtheriae* was identified from nasopharyngeal swab as well as throat swab. Others were identified only from nasopharyngeal swabs.

DTP3 coverage was the coverage at the village level where the carriers were living.

Chapter 6 Table 4. The associations between *C. diphtheriae* carriage and potential risk factors

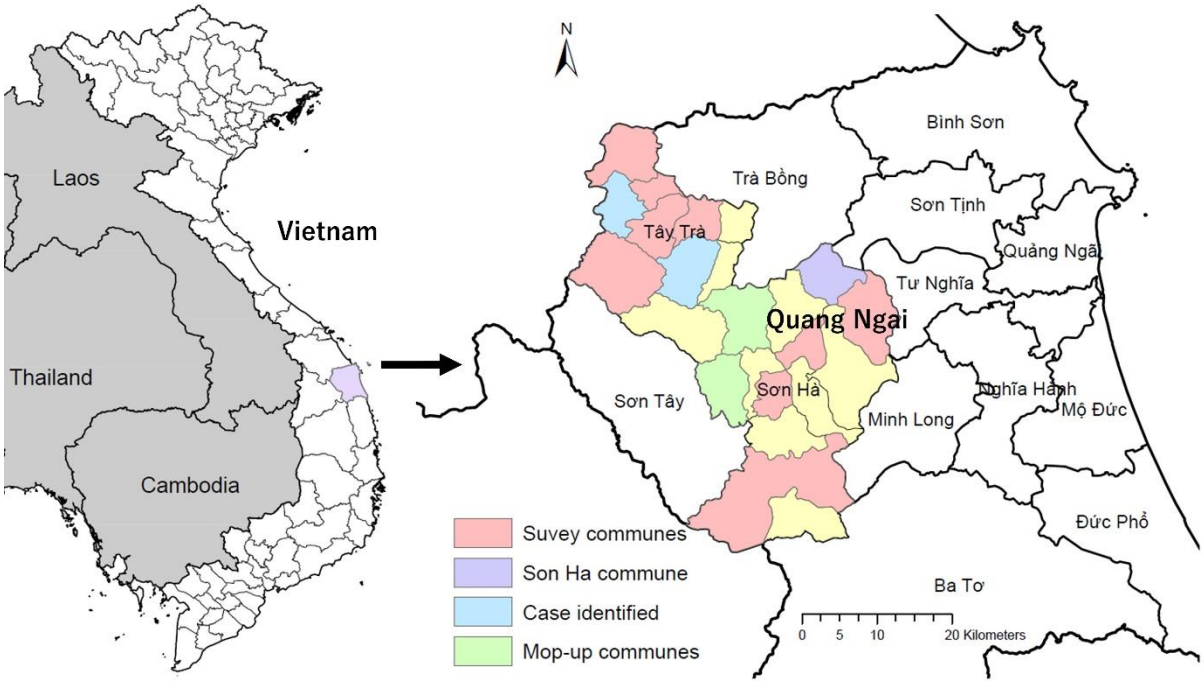
		Carriage				
Risk factor		**positive**		**negative**		**Fisher's exact test**
		n	%	n	%	p-value
Age group	≤5 yr	12	4.5%	257	95.5%	<0.01
	6-17 yr	10	3.1%	314	96.9%	
	18-40yr	5	1.0%	518	99.0%	
	41-55yr	0	0.0%	102	100.0%	
Sex	male	10	1.6%	605	98.4%	0.18
	female	17	2.8%	584	97.2%	
DTP1 (≤10years)	0 dose	5	4.5%	107	95.5%	>0.99
	≥1 dose	16	4.5%	336	95.5%	
DTP3 (≤10years)	<3 doses	5	4.3%	111	95.7%	>0.99
	≥3 doses	16	4.6%	332	95.4%	
Diphtheria antibody	<0.1 IU/ml	11	1.7%	630	98.3%	0.24
	≥0.1 IU/ml	16	2.8%	559	97.2%	
School	not attended	17	1.8%	906	98.2%	0.12
	attended	6	2.1%	283	97.9%	
Dormitory	not staying	23	2.2%	1,035	97.8%	0.77
	staying	4	2.5%	154	97.5%	
Sharing bed	no	4	2.7%	143	97.3%	0.56
	yes	23	2.2%	1,037	97.8%	
Household size	≤4 persons	13	2.2%	585	97.8%	>0.99
	>4 persons	14	2.3%	604	97.7%	
Bathing	< once/day	0	0.0%	72	100.0%	0.40
	≥once/day	27	2.4%	1,117	97.6%	
Handwashing	<3/day	4	1.6%	247	98.4%	0.11
	≥3/day	18	3.9%	445	96.1%	
Livestock or pet animal	no	24	2.7%	866	97.3%	0.08
	yes	3	0.9%	323	99.1%	
		positive		**negative**		**t-test** *p-value*
Mid upper arm circumference (MUAC) (cm)	mean (SD)	15.0 (1.77)		14.8 (1.33)		0.16
Age (years)	mean (SD)	7 (9.7)		20 (14.3)		<0.01
log-transformed IgG level	mean (SD)	-2.2 (2.1)		-2.4 (1.2)		<0.01

Chapter 6 Table 5. The association between *C. diphtheriae* carriage and anti-diphtheria toxoid IgG levels adjusted for age and assessed by logistic regression, and the association between IgG levels and mid-upper arm circumference (MUAC) adjusted for age by linear regression.

The association between carriage status and IgG	**crude Odds Ratio**	***p-value***	**adjusted Odds Ratio**	***p-value***
IgG level	1.44 (1.18 ,1.74)	<0.01	1.41 (1.15 ,1.74)	<0.01
Age (years)	0.94 (0.90 ,0.97)	<0.01	0.94 (0.90 ,0.97)	<0.01
The association between IgG and MUAC	**crude Coefficient**	***p-value***	**adjusted Coefficient**	***p-value***
MUAC(cm)	1.01 (0.99, 1.02)	0.43	1.02 (1.00, 1.03)	0.014
Age (years)	0.81 (0.71, 0.92)	<0.01	0.73 (0.64, 0.85)	<0.01

Crude coefficients and adjusted coefficients were transformed by anti- natural log for easy interpretation.

Chapter 6 Figure 1. Map of study areas and locations where the cases were identified before and during the study in Tay Tra and Son Ha districts in Quang Ngai Province, Vietnam



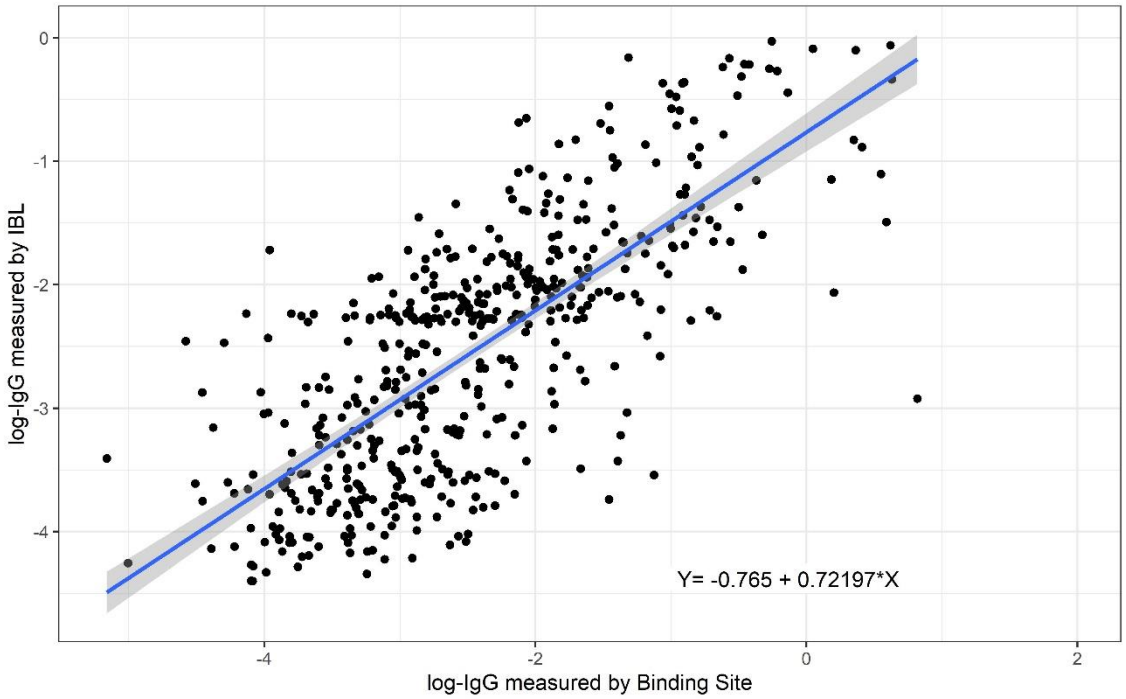
Red and purple: Ten communes were selected for this study.

Blue and purple: One laboratory-confirmed diphtheria case was reported between January and September 2019 in each of these communes.

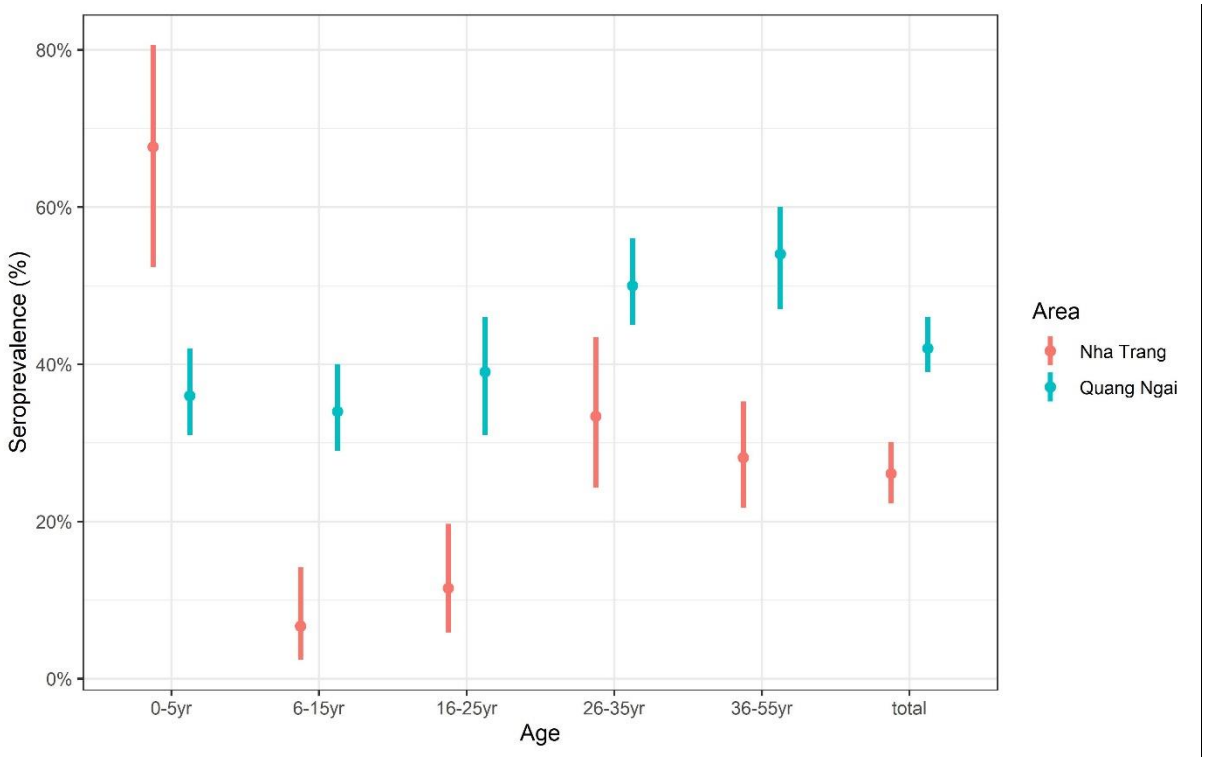
Purple (Son Ha commune): Twelve confirmed cases were reported in this commune within one month from the survey date, October 2019.

Green: Two communes were excluded from the selection process of this study as a mop-up vaccine campaign was conducted in 2018.

Chapter 6 Figure 2. The best-fitted linear regression line comparing log-transformed IgG concentrations measured by Binding Site and IBL ELISA assays



Chapter 6 Figure 3. Comparison of the age-stratified seroprevalence, the proportion of individuals with anti-diphtheria toxoid ≥ 0.1IU/ml, with 95%CI between Quang Ngai Province and Nha Trang city



	Nha Trang city	Quang Ngai province
1-5 years:	68% (95%CI:52-81)	36% (95%CI:31-42)
6-15 years:	7% (95%CI:2-14)	34% (95%CI:29-40)
16-25 years:	12% (95%CI:6-20)	39% (95%CI:31-46)
26-35 years:	33%(95%CI:24-43)	50% (95%CI:45-56)
36-55 years:	28% (95%CI:22-35)	54% (95%CI:47-60)
Total:	26% (95%CI:22-30)	42% (95%CI:39-46)

Quang Ngai's seroprevalence was not weighted by population for this comparison.

Chapter 7: Validation and correction of IgG antibody tests against diphtheria toxoid measured by ELISA compared with neutralisation assay

Chapter overview

Although TNT is a gold-standard assay to measure anti-diphtheria toxoid IgG in human sera, ELISA is often used in a population-based seroepidemiological studies as a simple and low-cost alternative. In the series of research for this thesis, ELISA was used to quantify the seroprevalence in two populations in Vietnam. However, ELISA does not accurately detect low antibody levels, and ELISA is recommended to be validated by TNT. There are two cut-off thresholds for diphtheria antitoxin measured by TNT; 0.01 IU/ml and 0.1 IU/ml. Interpretation of the antitoxin levels is: if $\leq$ 0.01 IU/ml (negative), individuals are susceptible; if equal to or greater than 0.01 IU/m and less than 0.1 IU/ml (equivocal), individuals have some degree of protection; and if $\geq$ 0.1 IU/ml (positive), individuals have long-term protection against diphtheria infection.

This chapter aims to validate the ELISA measurements used in this thesis compared with TNT measurements.

Serum samples were collected in one survey area in an urban setting, while dried blood spots (DBS) were collected during another survey in a rural area. DBS requires a small volume of samples by finger prick and does not require a facility for processing or storage; therefore, it is a valuable method for survey in resource-limited settings.

Furthermore, this chapter aims to confirm the validity of the DBS as a field-friendly alternative sample collection method for measuring the anti-diphtheria toxoid IgG in resource-limited settings, and its serological results are comparable with the results obtained from serum samples.

A seroepidemiological survey is helpful in LMICs as they often have difficulty controlling infectious diseases. In these countries, TNT is often unavailable, or resources are inadequate to test all samples using TNT. Therefore, this chapter proposes two methods to estimate the reliable seroprevalence of diphtheria in the population based on ELISA measurements with a parallel comparison of the paired measurements of TNT and ELISA in a subset of samples. One method is to identify the optimal cut-off values in ELISA corresponding to the thresholds of TNT. Another is to estimate the proxy TNT measurements in an individual sample based on available ELISA results and linear association between ELISA and TNT, considering the uncertainty.

Chapter summary

We collected 96 serum samples and DBSs collected in Nha Trang city were tested by both ELISA and TNT; their results were compared for the validation study. The diagnostic performance of ELISA with two cut-off values (0.1 and 0.01 IU/ml) compared to TNT was assessed by measuring sensitivity, specificity, kappa coefficient, and area under the curve (AUC). The results suggested that the seropositive and seronegative classified by the two assays agreed when the seropositive was defined above the cut-off value of 0.1 IU/ml. If the cut-off value of 0.01 IU/ml was used, the classification of the seropositivity in individuals did not agree between ELISA and TNT. ELISA results of DBS were agreed upon and correlated with ELISA results of serum samples, and DBS was confirmed to be a good alternative for serum samples.

Receiver operating characteristic (ROC) curve analysis identified the optimal cut-off values for ELISA corresponding to two TNT thresholds. In serum samples, 0.06 IU/ml and 0.064 IU/ml of ELISA corresponded to the TNT values 0.01 and 0.1 IU/ml, respectively. In DBS samples, 0.04 IU/ml and 0.105 IU/ml of ELISA were equivalent to TNT values of 0.01 IU/ml and 0.1 IU/ml, respectively. These cut-off thresholds could be potentially used to measure seroprevalence in a population where the TNT assay is unavailable. Instead, the samples falling into the equivocal results, e.g., between 0.06 IU/ml and 0.1 IU/ml in the ELISA test, could be re-evaluated by TNT (if available) to confirm the antibody levels of these samples.

A multiple imputation approach was applied to estimate the TNT measurement for individual samples by reconstructing the distribution of nine discrete TNT values based on the linear association between paired results of ELISA and TNT assays. The overall and age-stratified seroprevalence was estimated by using 1,000-times imputed data incorporating the uncertainty of the data. The seropositive proportion in the population and seropositive distribution pattern by age were well described using this method, although the true prevalence level when a cut-off value of 0.01 IU/ml was used was uncertain.

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RESEARCH PAPER COVER SHEET

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SECTION A – Student Details

Student ID Number	lsh422077	**Title**	Dr.
First Name(s)	Noriko		
Surname/Family Name	Kitamura		
Thesis Title	Understanding factors contributing to outbreaks of diphtheria and implications for vaccination policy in Viet Nam		
Primary Supervisor	Paul Fine		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Epidemiology and Infection
Please list the paper's authors in the intended authorship order:	Noriko Kitamura, MD, MPH Akira Endo, MD, PhD Lien T. Le, MSc Hung T. Do, MD, PhD Yoshio Mori, PhD

	David Litt, PhD Androulla Efstraciou, PhD Norman K. Fry, PhD Michiko Toizumi, MD, PhD Lay-Myint Yoshida, MD, PhD
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I have planned and designed a research and conducted a data collection an analyses with other co-authors.

SECTION E

Student Signature	Noriko Kitamura [redacted]
Date	1 Sep 2022

Supervisor Signature	Michiko Toizumi [redacted]
Date	21 September 2022

Introduction

Diphtheria is caused by toxin-producing strains of *Corynebacterium* species, mainly *C. diphtheriae* and *C. ulcerans,* and occasionally *C. pseudotuberculosis* (153). Diphtheria has almost been eliminated in Western countries; however, it is still endemic in low- and middle-income countries (LMICs) (38). Of note, over 90% of the total reported cases worldwide were in South and Southeast Asia in the late 2010s (38). Recent large-scale outbreaks in Rohingya refugee camps in Bangladesh, Venezuela, and Yemen highlighted the potential threat of diphtheria in many parts of the world (49, 55, 86).

Diphtheria incidence is attributed to low vaccine coverage (206). Seroepidemiological assessment of a population's susceptibility to diphtheria is important to estimate transmission potential in the population and to evaluate vaccination programmes. However, seroepidemiology is underused in LMICs due to the required costs and resources (233). The gold-standard method for measuring the level of functional IgG neutralising diphtheria toxin is the VERO cell TNT, which can be calibrated to report results in internationally recognised IU/ml (59). According to the current WHO laboratory manual, an individual's serum IgG level measured by TNT is classified as either ≥ 0.1 IU/ml (long-term protection), 0.010.1 IU/ml (some degree of protection), or < 0.01 IU/ml (susceptible) (205).

As TNT is time-consuming and requires facilities for cell culture, several alternative methods, including ELISA, have been used for seroepidemiological surveys as an simple, fast, and low-cost alternative (59). DBS on filter paper is also a simple and low-cost method for collecting, transporting, and storing samples in resource-limited settings without requiring on-site facilities for serum separation and a cold chain for transport and storage (233). DBS collected by finger prick is minimally invasive and collects a small blood volume, which is also an advantage for studies targeting young children, such as an assessment study for child immunisation programmes (234). Antibodies collected via DBS are stable for about 1 week at room temperature and for longer in a freezer (234, 235). Schou et al. reported a good linear correlation between anti-diphtheria toxin antibodies (using TNT) in serum and anti-diphtheria toxoid IgG (using ELISA) in DBS in 1987 (220). Therefore, ELISA measurements of DBS samples could be a suitable method for seroepidemiological studies in LMICs.

However, ELISA measurements do not necessarily correspond to TNT measurements, especially when anti-diphtheria toxoid IgG concentration is low (224, 236). Previous validation studies had limitations as they used samples with high titre (> 0.1 IU/ml) and did not distinguish between the equivocal (some degree of protection) and negative (susceptible) sera (205, 223, 237, 238). Anti-diphtheria antibody levels in an individual or population must be measured accurately to monitor immunogenicity of vaccines or waning of immunity to provide recommendations for vaccination policy (236). This study proposed several methods to estimate the proxy protection levels in the population with available ELISA measurements.

First, this study assessed the diagnostic performance of a commercial ELISA test (when used with serum and DBS samples) compared with TNT. Second, this study aimed to identify the optimal cut-off values in ELISA that yielded the most similar results to TNT with standard cut-off values of 0.01 IU/ml and 0.1 IU/ml, to distinguish between individuals with long-term protection, some degree of protection, or no protection based on ELISA. Third, this study aimed to estimate TNT measurements (using a method based on multiple imputation) in a dataset from a recent seroepidemiological survey in Vietnam that included only ELISA test results to more accurately quantify a population's level of protection against diphtheria.

Methods

Sample collection

An age-stratified cross-sectional seroprevalence survey was conducted in Nha Trang city, Vietnam, in 2017. In total, 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 (212). Of the 510 participants, 100 were randomly selected and recruited in 2019 to compare TNT and ELISA assays. Finally, two types of specimens, serum and DBS, were collected from 96 individuals and were available for the parallel comparison. The required sample size for comparing values in paired samples was identified based on the sample size calculation for the Bland-Altman method (α=0.05, power =80%, different standardised agreement limit = 2.5) (239) and justified by the sample size used in the previous study (220).

Whole blood (2 ml from participants younger than age 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, Vietnam). Whole blood was applied on Whatman 903 protein saver cards (#Z761575) until blood saturated a 0.5-inch diameter circle on the card and was allowed to dry, following the standard sample collection and storage method for DBS recommended by the US Centers for Disease Control and Prevention (214, 215). Both types of samples were transported to the Pasteur Institute in Nha Trang on the day of sample collection and stored at 4 °C. Serum samples were stored in a –80 °C freezer immediately after processing. DBS were punched out with a 6-mm-hole punch, placed in Eppendorf tubes, and stored in a -80 °C freezer until testing. Ethical approval was obtained from the Vietnamese MoH and the London School of Hygiene and Tropical Medicine ethical review boards (IRB-VN01057-27/2015, LSHTM Ethics ref: 17518/17913).

Laboratory assay

Anti-diphtheria toxoid IgG antibody levels were measured in serum and DBS samples using a commercial diphtheria ELISA kit (IBL, Germany, RE56191) in Vietnam. It was estimated that 5 µl of serum was absorbed in each 6-mm-diameter disc of the Whatman 903 card (214, 215, 219, 221, 235, 240). Each 6mm-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 performing ELISA. Elution buffer comprised phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween20 and 1% (w/v) skim milk (221). 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.

Frozen sera were transported from Vietnam to the UK to determine the concentration of diphtheria antitoxin in the sera by TNT at the Respiratory and Vaccine Preventable Bacteria Reference Unit, UK Health Security Agency (a WHO Collaborating Centre for reference and research on diphtheria). The VERO cell TNT assay is based on the capacity of diphtheria toxin to cause mammalian cell deaths and the neutralisation of this effect by diphtheria antitoxin antibodies when present in serum specimens. The serum anti-diphtheria toxoid IgG concentration was determined at the first dilution level in which VERO cells survived for 48–72 hours after being mixed with diphtheria toxin and serum specimens containing antitoxin antibodies. The lowest quantifiable IgG level by TNT is 0.008 IU/ml. TNT assay processing ten times twofold dilution of sera sample could take the values of <0.008, 0.008, 0.016, 0.032, 0.064, 0.128, 0.256, 0.512, 1.024, and 2.048 IU/ml. Individual serum IgG level

measured by TNT was interpreted as ≥0.1 IU/ml (long-term protection), 0.01–0.1 IU/ml (some degree of protection), or < 0.01 IU/ml (susceptible) (205).

Statistical analysis

The 96 samples that had both TNT and ELISA results were used to evaluate the accuracy and agreement of the two measurements in sera and DBS. If TNT measurements of IgG were lower than the lowest detection level (0.008 IU/ml), 0.004 IU/ml (one-half of 0.008) were imputed following to a method used in the previous study (224, 237). First, individual serum IgG levels measured by TNT and ELISA were classified as ≥ 0.1 IU/ml (positive), 0.01-0.1 IU/ml (equivocal), or < 0.01 IU/ml (negative) to evaluate the accuracy and agreement between the two methods. 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) to evaluate the agreement between the two measurements. Because the cut-off threshold recommended for ELISA was tenfold higher than that of TNT in some commercial kits (236), the number of samples classified as seropositive and seronegative using the 0.01 IU/ml cut-off in TNT and 0.1 IU/ml cut-off in ELISA were also compared for evaluation of their agreement. Sensitivity, specificity, and area under the receiver operating characteristic (ROC) curve (AUC) with 95% CI were calculated to assess the diagnostic accuracy (241, 242). The estimated AUC was considered an aggregate measure of the accuracy of ELISA compared with TNT. 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) (242, 243). Cohen's kappa coefficients were measured with 95% CI to evaluate the diagnostic agreement between TNT and ELISA. The kappa coefficient was interpreted as poor (<0.2), fair (0.210.40), moderate (0.41–0.60), good (0.61–0.80), and very good (0.81–1.00) (244).

The 96 samples that had both TNT and ELISA results were used to examine the association between IgG measurement in TNT and ELISA after the values were $\log_{10}$-transformed. Pearson's correlation coefficients were estimated with 95% CI to examine the association between TNT and ELISA measurements (245). Lin's concordance-correlation coefficients were calculated with 95% CI to assess the reproducibility of the test (246). A coefficient > 0.9 was interpreted as very good, and > 0.8 was interpreted as good reproducibility (224, 246).

Second, the optimal ELISA cut-off values for the classification of long-term protection (IgG ≥ 0.1 IU/ml in TNT), some degree of protection (IgG 0.1–0.01 IU/ml in TNT), and susceptible (defined as IgG levels < 0.01 IU/ml in TNT) were determined by ROC curve analysis (247). The point with maximum values of the Youden index, defined as *J* = sensitivity + specificity - 1, on the ROC curve was determined as the optimal cut-off point. The R package *pROC* was used for conducting this analysis (248, 249). The sensitivity and specificity of the new optimal cut-off values were calculated to confirm their diagnostic accuracy.

Third, a statistical method based on the multiple imputation approach was applied to reconstruct the distribution of nine discrete TNT values (from 0.004 IU/ml to 1.024 IU/ml, excluding 2.048 IU/ml as no samples took this value) and estimate TNT measurement in sera collected in the population-based survey in Vietnam in 2017. The multiple imputation approach is a technique to analyse the dataset with missing data. In this study, 96 TNT values were available in the dataset; however, TNT values for the remaining samples collected during the survey were missing. Therefore, the multiple imputation-based approach was applied to estimate the remaining TNT values and 'true' seroprevalence in the population. Using the observed linear association between IgG values measured by ELISA and TNT in the 96 reference samples, a multiple imputation generated 1,000 imputed values of TNT from ELISA measurements in each sample that did not contain TNT measurement (*N* = 510). The 95% CI for pooled estimates of seropositive proportions based on imputed data was calculated by Rubin's rule (250). Detailed methods are described in the supplementary material.

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: 1) using original ELISA measurements with standard cut-off values (0.1IU/ml and 0.01IU/ml), 2) using original ELISA measurements with the optimal cut-off values determined by ROC curve analysis, and 3) using estimated TNT measurements by multiple imputation-based method with standard cut-off values (0.1IU/ml and 0.01IU/ml). Statistical analyses were conducted using STATA15 and R software (225, 249).

Results

IgG levels in TNT and ELISA were categorised into three classes: ≥ 0.1 IU/ml (positive), 0.010.1 IU/ml (equivocal), and < 0.01 IU/ml (negative). When testing 96 samples of matched

sera and DBS, 40 sera were classified as negative by TNT, while only four sera and one DBS were classified as negative by ELISA. In contrast, 33 sera were classified as equivocal by TNT, while 68 sera and 69 DBS 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) were classified as negative (< 0.01 IU/ml) 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 (Chapter 7 Figure 1).

Sensitivity, specificity, AUC, and the Cohen's kappa coefficient of ELISA to detect protective titres (either > 0.1 IU/ml or > 0.01 IU/ml) against TNT results when using the same panel of 96 matched sera and DBS are summarised in Table 2. AUC (0.82 and 0.89 for serum and DBS, respectively) showed good performance in the ELISA test with a cut-off value of 0.1 IU/ml for both sample types; however, AUC showed fair or poor performance with a cut-off value of 0.01 IU/ml. Similarly, Cohen's kappa coefficients (0.63 and 0.75 for serum and DBS, respectively) showed a good agreement between the two tests with the cut-off value of 0.1 IU/ml; however, the agreement was fair or poor with a cut-off value of 0.01 IU/ml (244).

Correlation between TNT and ELISA measurements was assessed by Pearson's correlation coefficient (r), and the reproducibility of ELISA compared with TNT was assessed by Lin's concordance correlation coefficient (ρc). Pearson's correlation coefficients showed high correlations between TNT values and ELISA values (r = 0.74 in serum, and r = 0.80 in DBS); however, Lin's concordance correlation coefficients of ELISA against TNT (ρc = 0.7 in serum and ρc = 0.78 in DBS) were slightly below the level for good agreement in both serum and DBS samples (ρc = 0.8) (224, 246). The concordance between ELISA values measured in serum and DBS was very good (ρc = 0.95) (Chapter 7 Figure 1) (224, 246).

The optimal cut-off values for ELISA, which classified the individuals into long-term protection, some degree of protection, and susceptible against diphtheria, were identified by the point with maximum Youden index on the ROC curves. For serum samples, the cut-off values of 0.060 IU/ml and 0.064 IU/ml in ELISA corresponded to the cut-off values of 0.01 U/ml and 0.1 IU/ml in TNT, respectively. For DBS samples, the cut-off values of 0.044 IU/ml and 0.105 IU/ml corresponded to 0.01 IU/ml and 0.1 IU/ml in TNT, respectively (Chapter 7 Figure 2). The performance of the ELISA test expressed as sensitivity and specificity improved when the cut-off values were 0.060 IU/ml and 0.044 IU/ml for serum and

DBS, respectively, compared with the 0.01 IU/ml cut-off value for TNT. However, the performance of ELISA was not changed when the cut-off values were 0.064 IU/ml and 0.105 IU/ml for serum and DBS, respectively, compared with a 0.01 IU/ml cut-off value for TNT (Chapter 7 Table 2 and Chapter 7 Figure 2).

Using 510 serum samples collected in 2017 in a seroepidemiological study in Nha Trang city, Vietnam, we re-estimated the proportions of seropositive individuals based on the new optimal cut-off values for ELISA: 0.064 IU/ml corresponding to a TNT value of 0.1 IU/ml and 0.060 IU/ml corresponding to TNT value of 0.01 IU/ml. The overall estimated seropositive proportion of the population with a cut-off value of 0.064 IU/ml in Nha Trang city was 44% (95% CI: 40–48), and the proportion was 29% (95% CI: 25–33) with a cut-off value of 0.1 IU/ml. The estimated seropositive proportion of the population with a cut-off value of 0.06 IU/ml was 46% (95% CI: 42–51), and the proportion was 96% (95% CI: 94–97) with a cut-off value of 0.01 IU/ml. Age-stratified seroprevalence with 95% CI is plotted in Chapter 7 Figure 3.

Finally, anti-diphtheria toxin antibodies of TNT for 510 serum samples were estimated by applying the multiple imputation approach. We categorized 1,000 estimated anti-diphtheria toxin antibody levels in TNT into < 0.01 IU/ml, 0.010.1 IU/ml, and ≥ 0.1 IU/ml. Based on this classification, mean overall seroprevalence and age-stratified seroprevalence by 5-year age band were calculated for each cut-off value of 0.1 IU/ml and 0.01 IU/ml. The pooled estimate of seroprevalence based on the imputed data with a cut-off value of 0.1 IU/ml was 20% (95% CI: 15–24), which was similar to the original data (29%). The pooled estimate of seroprevalence with a cut-off value of 0.01 IU/ml was 65% (95% CI: 60–70), which was much lower than the seroprevalence measured in the original ELISA data (96%) (Chapter 7 Figure 4). 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 (Chapter 7 Figures 3 and 4). All three analyses suggested that 0.1 IU/ml by ELISA is a reasonable cut-off value to identify individuals with long-term protection.

Discussion

This study aimed to validate a commercial ELISA kit for measuring the anti-diphtheria toxoid antibody compared with TNT in serum and DBS samples, including a low titre (< 0.1 IU/ml). The diagnostic performance of ELISA, evaluated by AUC and kappa coefficient, in serum

and DBS samples compared with TNT was good when the cut-off value was 0.1 IU/ml but was not adequate when the cut-off value was 0.01 IU/ml. There were good correlations between ELISA values in serum or DBS samples and TNT values as aggregated data, evaluated by Pearson's correlation; however, one-to-one concordance between paired values was not confirmed as good when evaluated by Lin's concordance correlation coefficient. Referencing TNT, ELISA using DBS had better diagnostic performance indicators than ELISA using serum samples. This might be due to reduced factors influencing antibody concentrations during sample processing when separating sera, such as haematocrit levels or haemolysis, although the specific reason was not apparent. ELISA measurements of DBS samples and serum samples were well-correlated and agreed with each other. These results suggest that DBS is a preferred alternative to serum samples.

Another aim of this study was to classify IgG levels as some degree of protection and no protection more accurately by adjusting ELISA cut-off threshold corresponding to TNT 0.1 IU/ml and 0.01 IU/ml. The two optimal cut-off values in ELISA-analysed serum samples, 0.060 IU/ml and 0.064 IU/ml, were similar. This might be because the ELISA system detected IgG, which could not neutralise diphtheria toxin, and this unspecific IgG increased the total concentrations of IgG from 0.01 IU/ml (205). The close proximity of the two cut-off values might have occurred by chance due to the small sample size. Investigating the ROC curve in Chapter 7 figure 2 (top right), another potential cut-off value for serum which is corresponding to TNT 0.1 IU/ml appeared to exist with a very similar Youden index. If that value were applied, the optimal cut-off value would be quite different. The optimal cut-off threshold of long-term protection in DBS samples was 0.105 IU/ml, which was nearly equal to the standard threshold of 0.1 IU/ml in TNT. This result suggested that ELISA performed on DBS samples could provide a proxy protection level in the population that is estimated based on TNT when using a cut-off value of 0.1 IU/ml. Suppose the same ELISA kit is used for the seroepidemiological survey; seroprevalence could be estimated using each cut-off value corresponding to the standard cut-off threshold in TNT as a reference when TNT is unavailable. If TNT is available, the samples fall into ambiguous results, in this study, ELISA measurements between 0.06 IU/ml and 0.1 IU/ml could be re-evaluated by TNT assay, as recommended by WHO (205). Our study used only one commercial ELISA kit. As each ELISA kit has a different level of correlation with TNT, the optimal cut-off values are not generalisable to other commercial ELISA kits (205, 236).

The third aim of this study was to estimate an individual's anti-diphtheria toxin antibody level more accurately based on available ELISA results. While ROC analysis identified the optimal threshold for ELISA considering continuous ELISA values over the binary categories of TNT (positive or equivocal, equivocal, or negative), another approach estimated each individual's TNT measurement based on continuous ELISA and TNT measurements. The previous study used linear or quadratic regression models to predict TNT measurements from the results of ELISA or other serological methods (237). Meanwhile, this study applied a multiple imputation approach based on linear regression, which also considers the uncertainty of the association to estimate the actual TNT value for each individual. The pooled seroprevalence estimates suggested that about one-third of the population was susceptible to diphtheria in Nha Trang city, while the susceptible proportion was estimated to be only 4% of the population based on the original ELISA results. Furthermore, the pooled estimates of seroprevalence obtained by the multiple imputation approach were lower than the estimates based on the original ELISA values in all age strata. The estimated seroprevalence was reasonable and consistent with the Vietnamese context in which small-scale diphtheria outbreaks continue to occur, which suggested that susceptible individuals remain in the population (17, 19). The multiple imputation-based approaches required only some 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 TNT measurements 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 the TNT assay method was well controlled. Ninety-six reference samples used for parallel comparison between TNT and ELISA had a skewed distribution towards low concentration of IgG; 40 (41%) of the samples were < 0.01 IU/ml, and 73 (76%) were < 0.1 IU/ml measured by TNT (Table 1). Although the samples with low values were ideal for addressing the problem of ELISA assay, the results may differ in other datasets with different distributions. For negative TNT results, 0.004 IU/ml was arbitrarily used, although the actual antibody levels might have varied. The analysis was conducted using a small sample size: anti-diphtheria toxin antibody values can be estimated more accurately with a larger sample size.

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 cut-off value of 0.1 IU/ml 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 in ELISA appears to underestimate the proportion of the susceptible population, and the use of this cut-off can be misleading. In diphtheria seroepidemiological surveys, testing a subset of samples via TNT could improve the assessment of the susceptibility against diphtheria at the population level.

Chapter 7 Table 1. Comparison of TNT and ELISA values in three categories <0.01 IU/ml, 0.01-0.1 IU/ml, and ≥ 0.1 IU/ml

TNT (IU/ml)	ELISA Serum (IU/ml) <0.01	0.01-0.1	≥0.1	Total
<0.01	4	34	2	40
0.01-0.1	0	28	5	33
>=0.1	0	6	17	23
Total	4	68	24	96

TNT (IU/ml)	ELISA DBS (IU/ml) <0.01	0.01-0.1	≥0.1	Total
<0.01	1	39	0	40
0.01-0.1	0	27	6	33
>=0.1	0	3	20	23
Total	1	69	26	96

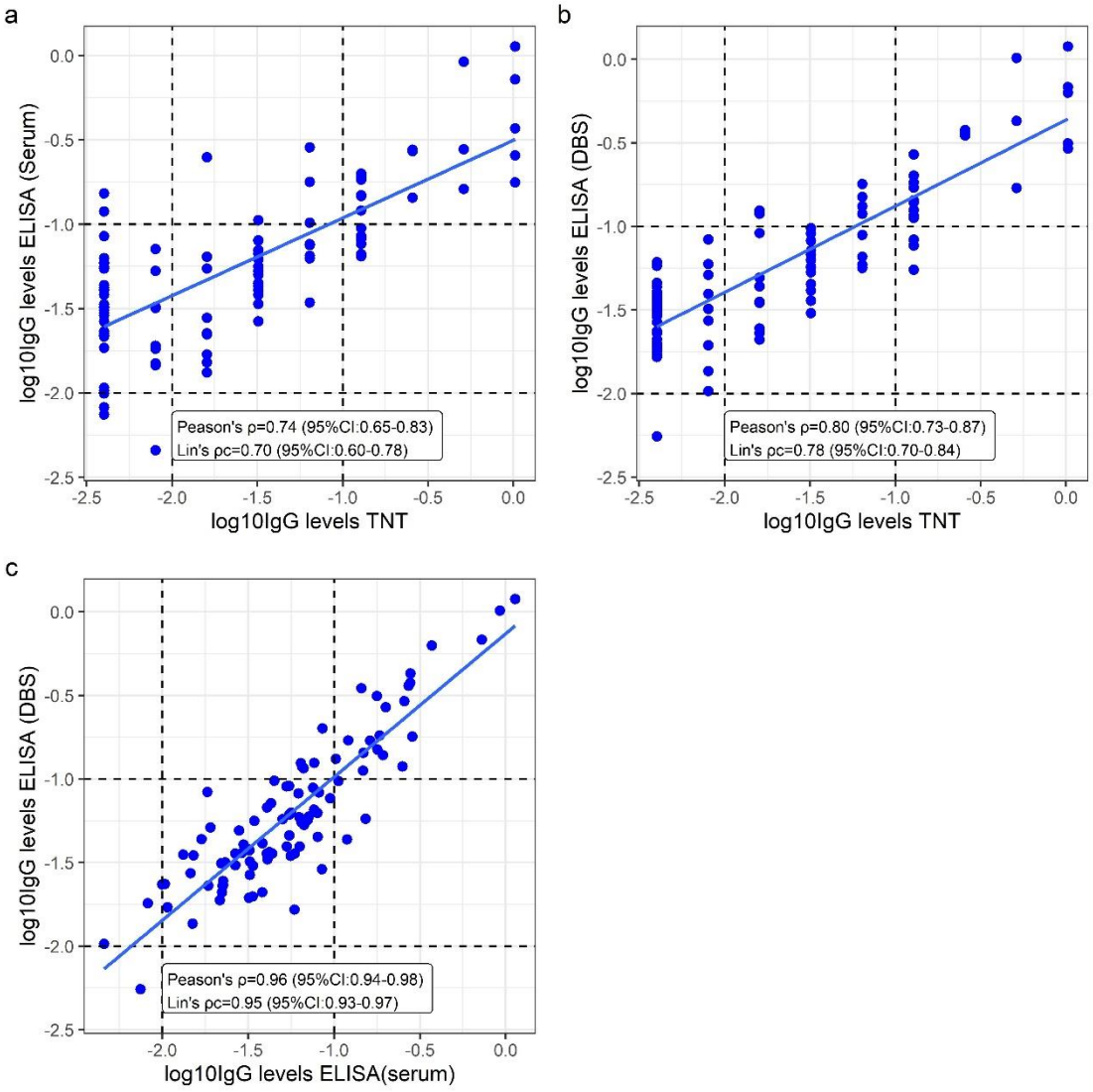
ELISA Serum (IU/ml)	ELISA DBS (IU/ml) <0.01	0.01-0.1	≥0.1	Total
<0.01	1	3	0	4
0.1-0.01	0	63	5	68
>=0.1	0	3	21	24
Total	1	69	26	96

Chapter 7 Table 2. Sensitivity, specificity, Cohen's kappa index, area under the receiver operator characteristics curve (AUC) of ELISA in different types of samples compared with TNT, a gold standard method for anti-diphtheria toxin antibody measurement assay.

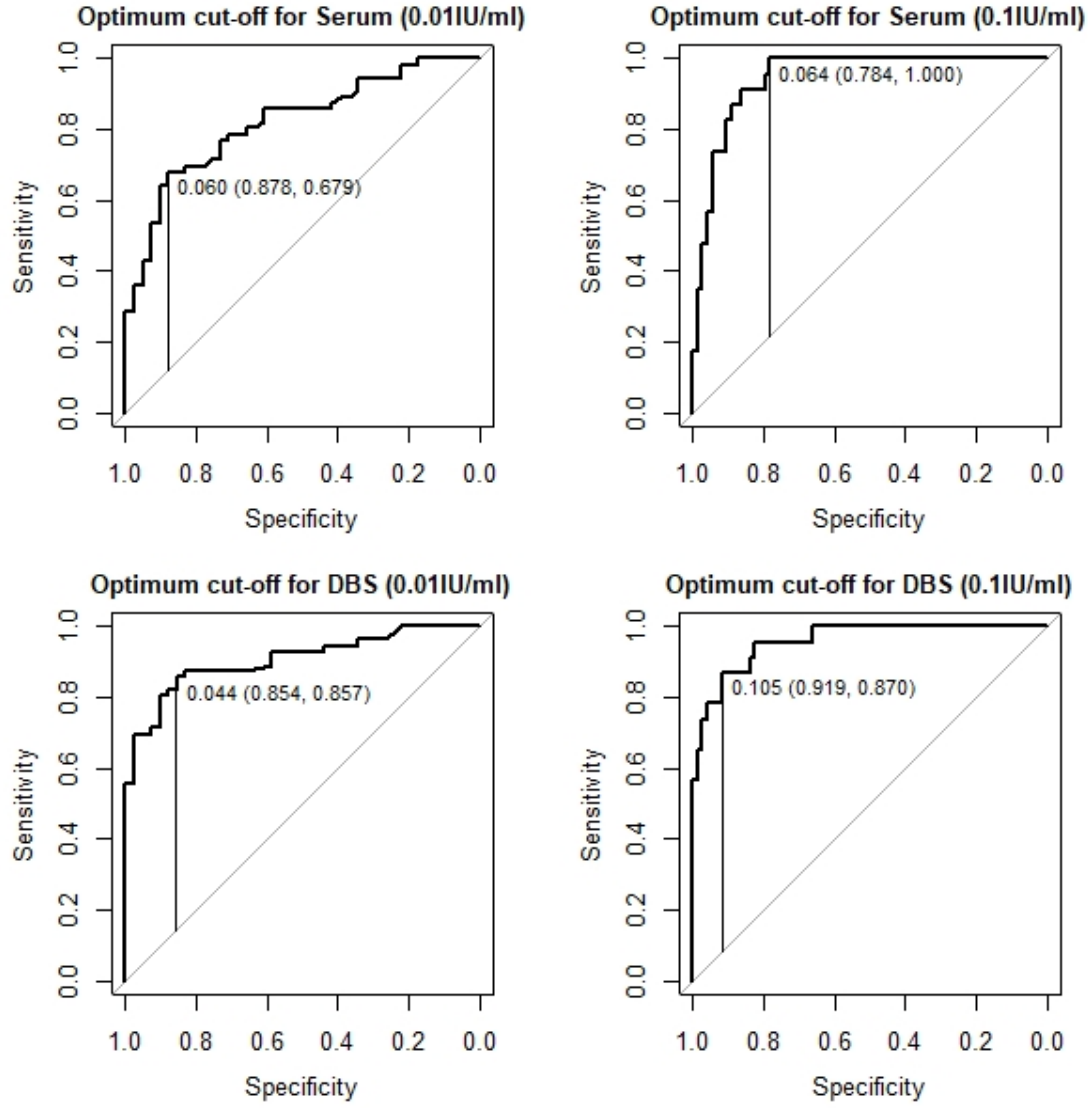
	TNT Cut-off	ELISA cut-off	
		Serum 0.01IU/ml	DBS 0.01IU/ml
Sensitivity	0.01IU/ml	1.00	1.00
Specificity		0.10	0.03
Kappa index		0.11(0.02,0.21)	0.03(-0.02,0.08)
AUC		0.55(0.50,0.6)	0.51(0.49,0.54)
	TNT Cut-off	ELISA cut-off	
		Serum 0.1IU/ml	DBS 0.1IU/ml
Sensitivity	0.01IU/ml	0.39	0.46
Specificity		0.95	1.00
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)
	TNT Cut-off	ELISA cut-off	
		Serum 0.1IU/ml	DBS 0.1IU/ml
Sensitivity	0.1IU/ml	0.74	0.87
Specificity		0.91	0.92
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)

	ELISA cut-off Serum	ELISA cut-off DBS 0.01IU/ml
Sensitivity	0.01IU/ml	1.00
Specificity		0.25
Kappa index		0.39(0.23,0.55)
AUC		0.63(0.38,0.87)
	ELISA cut-off Serum	ELISA cut-off DBS 0.1IU/ml
Sensitivity	0.1IU/ml	0.91
Specificity		0.92
Kappa index		0.78(0.58,0.98)
AUC		0.90(0.83,0.98)

Chapter 7 Figure 1. Comparison of the values of TNT and ELISA in serum (a), TNT and ELISA in DBS (b), and ELISA in serum and ELISA in DBS (c) with the equations of fitted lines, Pearson's correlation coefficient and Lin's concordance correlation coefficient

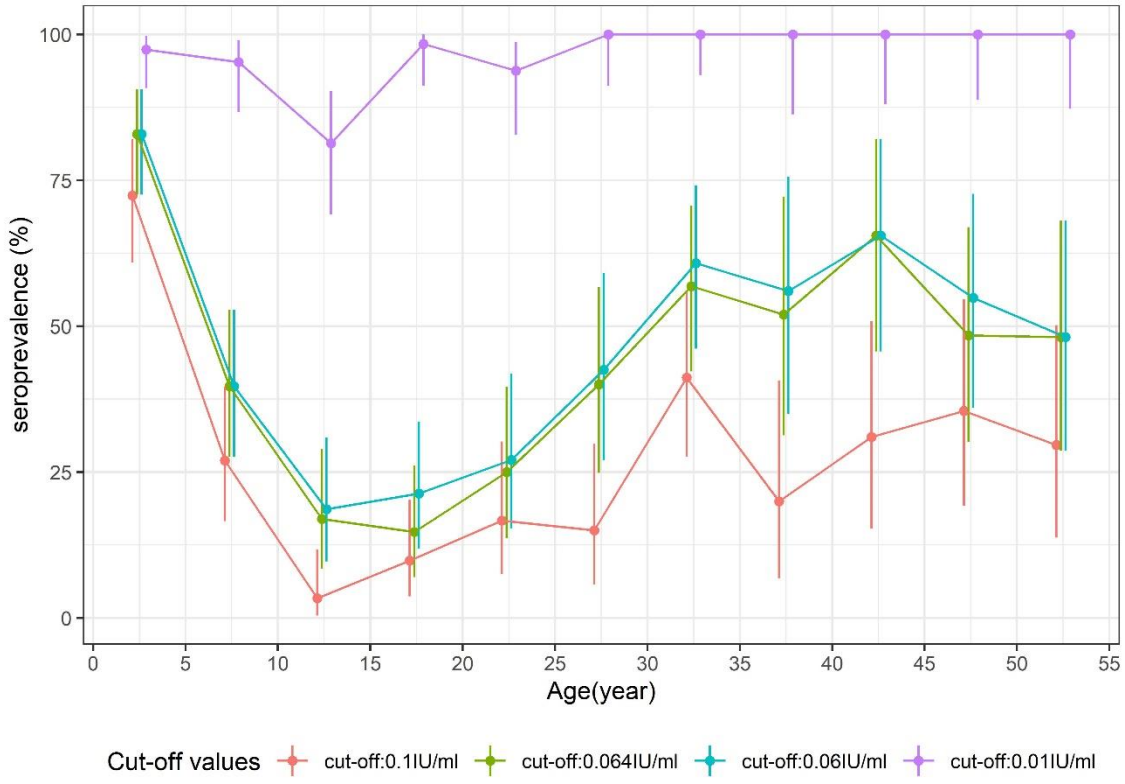


Chapter 7 Figure 2. Optimal cut-off values for ELISA in serum and DBS samples which classify individuals as susceptible (TNT < 0.01IU/ml) or long-term protected (TNT ≥ 0.1IU/ml)

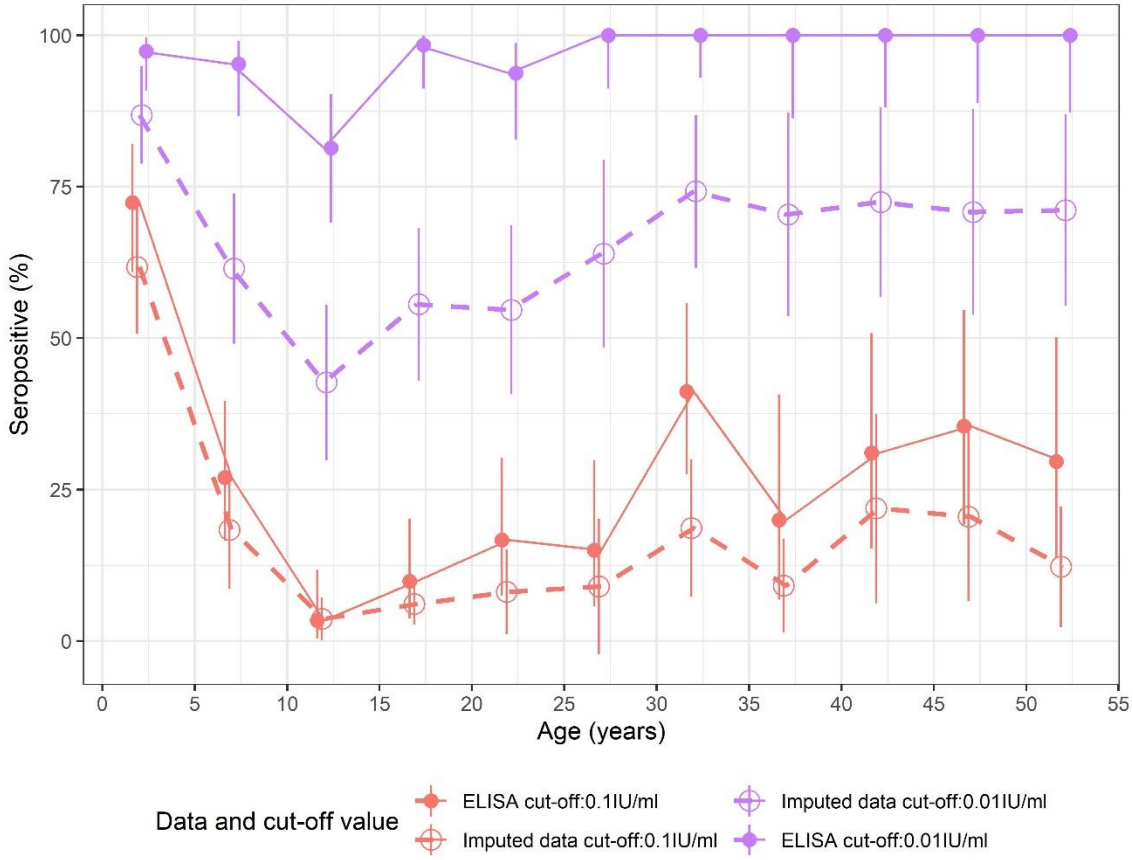


Each graph shows the "optimal cut-off value" and its ("specificity", "sensitivity") for ELISA using serum or DBS when each optimal cut-off value classifies seropositivity. The vertical line shows the Youden index for each ROC curve.

Chapter 7 Figure 3. Age-stratified seroprevalence of diphtheria in Nha Trang, Vietnam, in 2017, with 95% CIs classified by standard cut-off values, 0.1 IU/ml and 0.01 IU/ml, and obtained cut-off values, 0.064 IU/ml and 0.06 IU/ml, in ELISA using serum samples



Chapter 7 Figure 4. Age-stratified seroprevalence of diphtheria in Nha Trang, Vietnam, in 2017, with 95% CIs classified b standard cut-off values (0.1 and 0.01 IU/ml): 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.

Chapter 7 Supplementary Material

Methods:

Toxin Neutralization Test (TNT) is a gold-standard method for measuring anti-diphtheria toxoid IgG neutralizing capacity in serum IgG levels. ELISA is often used as an alternative for TNT that provides results measured on the same scale. Individual serum IgG measurement in TNT was interpreted as ≥ 0.1 IU/ml (considered as long-term protection), 0.01-0.1 IU/ml (some degree of protection), and < 0.01 IU/ml (susceptible) (205), but the IgG measurement in ELISA does not necessarily correspond to the measurement in TNT for the same sample, especially when IgG measurement in serum is low. In this study, a statistical method based on the multiple imputation approach was applied to estimate the IgG measurement in TNT for survey participants who only had ELISA test results (N = 510; hereafter referred to as the target dataset).

We first modelled the relationship between the measurements in the two tests using a reference dataset with both TNT and ELISA values, which was collected from the same cohort of survey participants but in a different year (N = 96). IgG measurement in TNT took nine discrete values, 0.004 (used as a proxy for any value < 0.008, following convention (224)), 0.008, 0.016, 0.032, 0.064, 0.128, 0.256, 0.512 and 1.024 IU/ml. We assumed that the ELISA measurement for a sample whose TNT measurement is *x* follows

$$y \sim N(ax + b, \sigma), \qquad \text{(S1)}$$

where *a* and *b* are constants specifying the linear relationship between the TNT and ELISA measurements and σ is the standard deviation of the residuals. Let p_k (k = 1, 2,…, 9) represent the relative frequencies of the TNT values in the target dataset. The ELISA values in the target dataset $Y = \{y_1, y_2, \ldots y_{510}\}$ is then expected to be independent samples from a mixture of normal distributions corresponding to the nine possible TNT values x_k (k = 1, 2,…, 9) with weights p_k. The likelihood of observing *Y* is therefore

$$L(Y) = \prod_{i=1}^{510} \sum_{k=1}^{9} p_k N(y_i | ax_k + b, \sigma). \quad \text{(S2)}$$

With the values for *a*, *b,* and σ fixed at the estimates from the linear regression analysis of the reference dataset, we estimated p_k by maximising the likelihood in Equation (S2) with the Limited-memory Broyden-Fletcher-Goldfarb-Shanno algorithm with box constraints (L-BFGS-B). As the number of samples in the reference dataset was small, mildly informative prior distribution was given to the weights p_k to prevent overfitting. We then imputed the missing TNT value for each sample in the target dataset using the conditional probability given the ELISA value *y*, i.e.

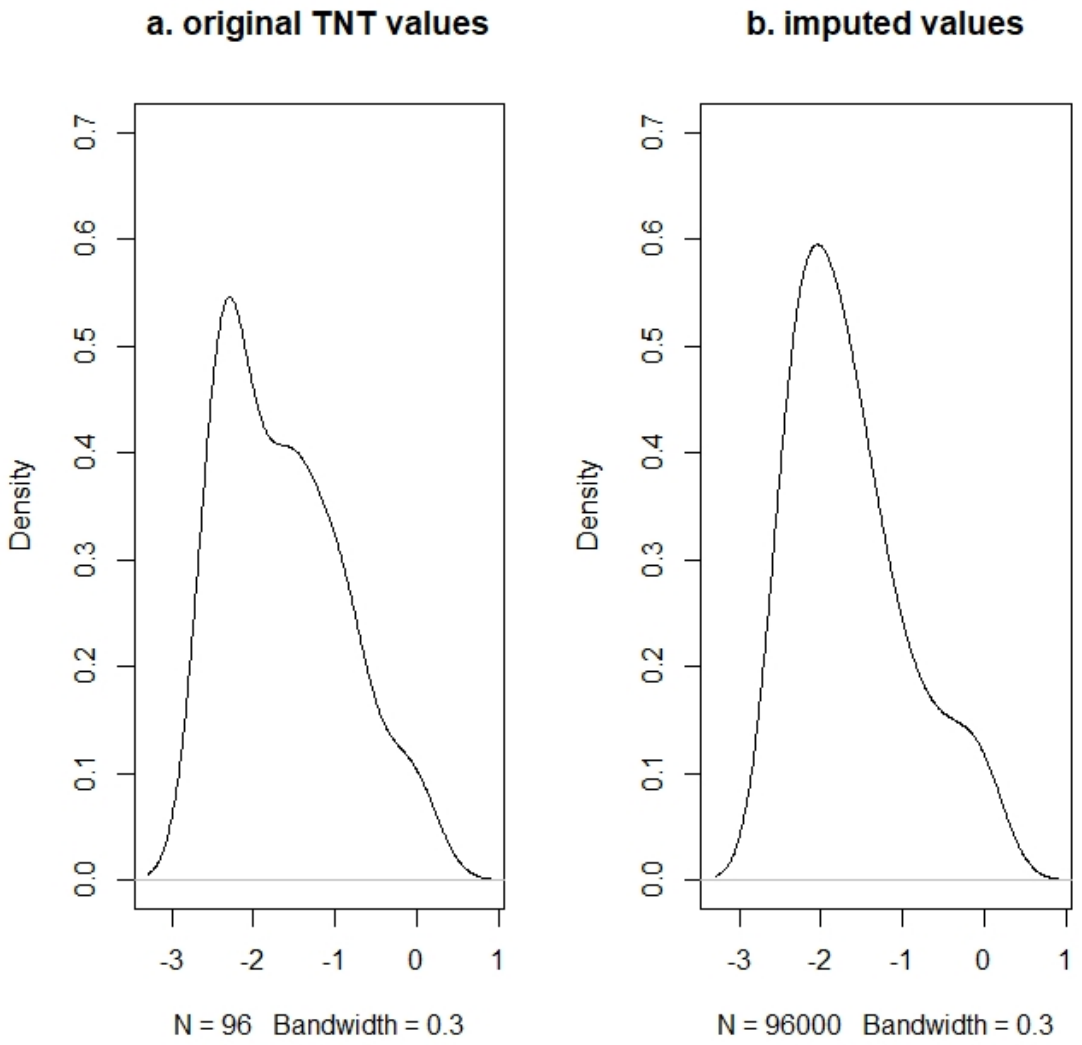
$$\Pr(x_k | y) = \frac{p_k N(y | ax_k + b, \sigma)}{\sum_{l=1}^{9} p_l N(y | ax_l + b, \sigma)}, \qquad (k = 1, \ldots, 9) \quad \text{(S2)}$$

to generate 1,000 multiple-imputation datasets. The pooled estimates of the proportions seropositive, defined as IgG level ≥ 0.1 IU/ml and IgG level ≥ 0.01 IU/ml, and their 95% confidence intervals were then calculated for 5-year-band age groups and compared with the seroprevalences based on the original IgG measurements in ELISA.

Regression coefficient *a*, intercept *b*, and standard deviation σ in Equation (S1) were estimated from the reference samples as *a* = -0.502*, b* = 0.461*,* and σ = 0.306.

To assess the performance of our reconstruction method, we compared the estimated TNT values from ELISA data of the reference samples with the actual TNT values. The distribution of imputed 1,000 datasets for the 96 reference samples was comparable between the reconstructed and measured TNT values (supplemental figure S1).

Chapter 7 Supplemental figure S1. Density distribution of 96 samples with TNT values (a) and imputed values (b).



The X-axis is the $\log_{10}$ scale of IgG concentration.

S1a and S1b showed similar distribution, which confirms the estimation method was valid.

Chapter 8: Overall discussion and conclusion

The reported number of diphtheria cases has been continuously declining globally since the introduction of the diphtheria toxoid vaccine. However, small outbreaks have continued to occur in Vietnam over the last 10 years. Several large diphtheria outbreaks have been observed in parts of the world in the latter half of the 2010s, especially in war zones and unstable societies where infant vaccination was disrupted. According to the commonly recognised facts about diphtheria, *C. diphtheriae* causes severe disease only in those who have not been vaccinated at all, those who have received at least one dose of the DTP vaccine have mild symptoms or are asymptomatic, and vaccinated individuals are thought to be protected from clinical diphtheria in general. It was also presumed that vaccinated individuals were protected from diphtheria for at least 10 years after vaccination (199). However, two children vaccinated with three doses of DTP or more died from diphtheria in Vietnam (Chapter 3). Their vaccination registration records were confirmed at the local health centres. The local authority confirmed the temperature of their cold chain in their electric log-record. This finding implies that the recent diphtheria outbreaks in Vietnam were caused not only by low infant vaccination coverage but also by the waning of vaccine-derived immunity. Better control strategies for diphtheria should be identified by studying the outbreaks and clarifying the population immunity and transmission pattern of *C. diphtheriae* in the endemic area. Therefore, a research series was conducted to understand the underlying mechanisms of the outbreak and to suggest appropriate vaccination policies in the country.

The first objective of the research was to describe the characteristics of the recent diphtheria outbreak in Vietnam. Seventy-three percent of the laboratory-confirmed cases were school-age children, which suggested that population immunity was low in this age group. Two children aged 7 and 13 years who received three doses or more of DTP died from diphtheria, which indicates that vaccine-derived immunity might have waned or that the vaccines they were given were ineffective. The local Vietnamese government confirmed that the cold chain was maintained in the areas, so that the vaccine should not have lost potency due to a broken cold chain. The average infant DTP3 coverage was 57% (95% CI: 53.3–61.2) in the communities where diphtheria cases were identified, which was significantly lower than the DTP3 coverage in the surrounding communities where no cases were identified (77% [95% CI: 74.9-79.0%], $p < 0.05$). This finding confirmed that DTP3 coverage was important to the transmission dynamics of diphtheria. Cases identified in the same school or geographic areas (with an epidemiological link) shared the same MLST type even though these cases were identified years after the previous case. This observation suggests that multiple strains were circulating in the study community and did not represent an

isolated imported case from a neighbouring country, such as Lao PDR, where diphtheria outbreaks were periodically reported. The outbreak investigation team identified the epidemiological link between the index case and other cases. However, they had difficulty tracking the links if the interval between the cases was long. This observation indicates that several generations of transmission might be maintained by asymptomatic carriers; either unvaccinated hosts infected with non-toxigenic strains or vaccinated hosts infected with toxigenic strains.

The second objective of the research was to assess the level of protection against diphtheria in the population. An age-stratified cross-sectional seroprevalence survey was conducted among 0–55-year old participants in 2017 in Nha Trang city, a well-developed and highly-vaccinated urban city in Vietnam. Seroprevalence was defined as the proportion of individuals whose anti-diphtheria toxoid antibody (IgG) levels in sera were ≥ 0.1IU/ml detected by ELISA (IBL) among the total participants in the survey. This survey revealed that overall seroprevalence in this population was 26% (95% CI:22–30). Age-specific seroprevalence plotted over age revealed a V-shape, which hit bottom at age 10 years. The lowest seroprevalence was 7% (95% CI:2–14) in the 6–15-year age group. This age pattern of seroprevalence explains why 73% of recent diphtheria cases in Vietnam belonged to the age group of 5–14 years. At the same time, the seroprevalence in adults (35–55 years) was also low, 28% (95% CI:22–35), which explained why the oldest laboratory-confirmed diphtheria case in Vietnam between 2013 and 2018 was 55 years old, and adult diphtheria cases were not rare in recent outbreaks in other countries. This survey clearly suggested that 30 years of infant vaccination programme without a school-entry booster dose has made a large proportion of the population susceptible, especially school-age children, by reducing the chance of natural exposure to the pathogen and waning of vaccine-derived immunity.

The third objective of the research was to estimate optimal booster dose intervals for DTP. The optimal timing of the booster dose was determined by the duration of protection after each vaccine dose. Therefore, the duration of protection was quantified based on the peak level of immune response and waning rate of immunity after different numbers of DTP doses. A systematic review was conducted to investigate publicly available serosurvey data to estimate the duration of protection after each vaccination. Among 15 cross-sectional seroprevalence studies in European countries, the estimated annual percentage decrease in the GMC of anti-diphtheria toxoid antibodies was 26%, 17%, and 7% per year after three, four, and five doses of DTP, respectively. The estimated duration of protection was 2.5 years, 10.3 years, and 25.1 years after three, four, and five doses of DTP, respectively. This result was the first estimate of the duration of protection of the anti-diphtheria toxoid IgG as a function of the number of doses of DTP vaccination. The estimated duration of protection

after each number of DTP vaccines was interpreted as the potential booster dose interval. Therefore, the obtained results could be considered as a reference to determine the optimal age for each booster dose; however, they do not consider the different types and combinations of vaccines (e.g., DTwP vs. DTaP vs. DTP-Hib-HepB) and the difference between an accelerated primary-dose schedule (e.g., 6, 10, and 14 weeks) and a delayed schedule (e.g., 3, 5, and 11 months). Therefore, application of the results requires careful consideration. A seroepidemiological study in Sub-Saharan African countries with only three primary doses may be a future research interest for investigating the optimal timing of the first booster dose in low-income countries.

For estimating the optimal school-entry booster dose timing in Vietnam, further analyses were conducted using longitudinal serological data collected in Nha Trang city. Based on two cross-sectional seroprevalence surveys conducted 2 years apart, waning rate and duration of protective immunity against diphtheria were estimated. The annual percentage decline of antibodies was 47% (95% CI:31–59) after four doses, and the duration of protection after four doses of DTP was estimated to be 4.3 years (95% CI:3.5–5.3). The estimates and significantly low seroprevalence among school-age children suggest that a school-entry booster dose should be implemented in Vietnam.

There was a significant difference between the two estimated durations of protection after four doses of DTP: 10.3 years based on European data and 4.3 years based on Vietnamese data. European survey data were cross-sectional, and each participant's vaccination history was unavailable, which was not ideal for estimating the waning of immunity. In contrast, the Vietnamese study followed the same participants for 2 years, and the analysis only included participants whose vaccination histories were confirmed by individuals or facility records. The European data included individuals up to 20 years old, while the Vietnamese data included individuals aged 1–7 years. The waning rate of immunity was assumed to be constant on a log scale in both analyses, although the waning rate appeared to differ by time since most recent vaccination. The different methods used for the analyses might have affected the findings. Although both methods have their limitations, it is more appropriate to use Vietnamese longitudinal data for decision-making on the optimal interval between booster doses in Vietnam. Given that the first DTP booster dose is scheduled at 18 months of age and the fourth DTP dose provides 4.3 years of protection, the second booster dose should be given at 6 years of age in Vietnam. Because school starts at the age of 6 years in Vietnam, a school-entry booster dose at age 6 is appropriate in the Vietnamese social system.

The fourth objective of the research was to measure the age-specific carriage prevalence and seroprevalence in diphtheria epidemic-prone areas. An age-stratified seroprevalence and carriage prevalence survey was conducted in Quang Ngai province, a rural farming community in Vietnam, where diphtheria cases had recently been reported. The overall weighted seroprevalence in Quang Ngai province was 52%, the seroprevalence of children aged 1–5 years was 43%, and the lowest seroprevalence (37%) was observed among children 6–17 years old. Symptomatic or asymptomatic infections occurred repeatedly among individuals older than school-age, as seroprevalence continuously increased among the population older than 17 years. The highest carriage prevalence was observed in the age group of 1–5 years (2.7%), followed by 6–15 years (2.6%), and carriage prevalence declined with age. Age-stratified seroprevalence and carriage prevalence were negatively correlated at the aggregated level because carriers were likely to be found among individuals whose anti-diphtheria antibody levels were low (210).

The immunity pattern by age significantly differed between Nha Trang City and Quang Ngai province. The seroprevalence of participants 1–5 years was 67% in Nha Trang city and 36% (unweighted) in Quang Ngai province. The administrative vaccine coverage in two communes in Nha Trang city in this age group was nearly 100%, except for 4 years old (59%), while at least 87% of the study participants aged 1–5 years received at least three doses of DTP in Quang Ngai province. Considering the difference in the vaccination coverage between the two populations, the seroprevalence among 1–5-year-old was low in Quang Ngai province. A potential cause of the low seroprevalence among 1–5-year-old in Quang Ngai province is that history of a fourth dose of DTP among participants was much higher in Nha Trang (62%) than in Quang Ngai (43%). This result suggests that the fourth dose of DTP received in the second year of life might have effectively boosted immunity in individuals and maintained adequate protection among children aged 1–5 years. Future serosurvey in a population with only three primary doses would reveal the booster effect on seroprevalence of the fourth dose in the age group of 1–5 years old.

The study found that 1.1% of the population were asymptomatic carriers of *C. diphtheriae*, one-third of which harboured a toxigenic strain. This prevalence was much higher than the prevalence in Europe measured in 2007–2008 (113). Carriage prevalence of toxigenic strain was nearly zero in Europe except for some countries in the former Soviet Union, which experienced a massive diphtheria outbreak in the 1990s. In contrast, the carriage prevalence in Indonesia in 2012 was 3% among children aged 1–15 years, and the carriage prevalence in the UK in 1971 was 1.2 %; both were similar to the prevalence in Vietnam (118). Because continuously high vaccine uptake reduces the carriage prevalence of toxigenic strains, this finding suggested that the vaccination uptake since the introduction of the vaccine in

diphtheria endemic areas, such as Vietnam and Indonesia, has been insufficient to eliminate toxigenic strains. Although DTP was one of the vaccines introduced in the early phase of the EPI programme, DTP introduction in Vietnam and Indonesia occurred about 40 years later than in European countries. Based on these observations, high vaccine coverage should be maintained for at least a few decades to eliminate diphtheria in Vietnam.

The fifth objective of the research was to identify the risk factors for bacterial carriage status, which can lead to clinical diphtheria. Carriers identified in Quang Ngai province and their biological and social factors were evaluated via logistic regression analysis. Young children were likely to be a carrier, but no other social or behavioural factors, such as school attendance, staying in the school dormitory, frequency of bathing and handwashing, sharing beds or utensils, or household size, were associated with carriage status. Low nutrition status, measured as MUAC, was associated with low immunity levels in individuals after adjusting for age; however, low nutrition status was not an associated with carriage status. This finding suggests that poor nutrition may prohibit seroconversion or reduce the magnitude of the immune response to the DTP vaccine in children. Malnutrition might be another reason for the low seroprevalence among children aged 1–5 years in Quang Ngai province, as the prevalence of acute and chronic malnutrition was critically high in the province.

In addition to the findings, useful information for elucidating potential mechanisms of recent diphtheria outbreaks was obtained by comparing the seroprevalence pattern by age in Nha Trang city, where no cases were reported for in the last decade, and Quang Ngai province, where 49 cases were reported between 2019 and 2020. Among 1–5-year old children, administrative DTP3 coverage was nearly 100% in Nha Trang city, while DTP3 coverage among study participants was 87% in Quang Ngai province. A significant difference in seroprevalence was observed between in the age group of 6-15 years and to the age group of 1–5 years: 7% in Nha Trang city and 34% (unweighted) in Quang Ngai province. Furthermore, the seroprevalence of the population above 15 years of age was lower in Nha Trang than in Quang Ngai. This finding suggests that the population in a well-vaccinated community has a lower protection level than an inadequately vaccinated population due to the loss of natural exposure by vaccine introduction and the waning of vaccine-derived immunity.

High infant vaccine coverage protected 1–5-year-old children in Nha Trang city. However, low infant vaccine coverage led to low immunity among children 1–5 years old in Quang Ngai province, predisposing them to becoming asymptomatic carriers. Children with low antibody levels are likely to become carriers and play a primary role in the transmission of *C.*

diphtheriae. However, these children do not manifest clinical symptoms of diphtheria because a low level of vaccine-derived immunity continues to protect them from symptomatic disease. An individual's immunity wanes over time, and their antibody levels decline to the lowest level at school-age. When children of school-going age are infected with *C. diphtheriae*, the hosts become symptomatic as their immunity is below the protection level. This phenomenon describes the mechanism of the recent diphtheria outbreaks in Vietnam and other LMICs that have not introduced multiple booster doses or whose vaccine coverages has been suboptimal. Myocarditis is one of the complications of diphtheria and is most commonly observed in teenagers (154). Myocarditis may lead to a high case fatality ratio because the highest proportion of patients are at the age at which most fatal complication occurs, while the pathogenicity of *C. diphtheriae* has not changed.

The last objective of this thesis was to validate the ELISA assay to detect accurate anti-diphtheria toxoid IgG compared with TNT. ELISA was used to measure the anti-diphtheria toxoid IgG levels in serum or DBS. The diagnostic performance of ELISA in serum and DBS with two cut-off values, 0.01 IU/ml and 0.1 IU/ml, was assessed compared with TNT measurement. Analyses showed that, if a cut-off value of 0.1 IU/ml was used, ELISA reliably classified individuals as seropositive in both serum and DBS, compared to TNT. We measured seroprevalence using a cut-off value of 0.1 IU/ml; therefore, the estimated population immunity in the two study areas should be reliable. In addition, results measured in serum samples and DBS samples were comparable.

According to the results of the research series, improved DTP3 coverage among infants and the introduction of a school-entry booster dose are recommended to control current diphtheria transmission in Vietnam. The cost-effectiveness of introduction of a booster dose in LMICs has been brought to a discussion as the number of reported diphtheria cases is not high. Historically, a decline in the incidence of diphtheria carriers has been attributed to vaccine introduction, which decreases the prevalence of toxigenic strains in the population (129, 130). Not only the primary-dose series, but pre-school, school-entry, and school-leaving booster doses protecting a wide range of age groups have been consecutively introduced in industrialised countries as the age of the cases shifts from young to old age after vaccine introduction (43). The introduction of an adult booster dose was discussed after large outbreaks occurred in the former Soviet Union, as adult patients accounted for two-thirds of the cases (43, 48). However, there is currently no clear evidence that an adult booster dose is necessary (38). High vaccine uptake, including booster doses protecting all age groups, plays a role in achieving low carriage prevalence of toxigenic strains in the upper respiratory tract in human hosts. Considering the costs of outbreak investigation and

response activities, including SIA targeting a broad age range in a large population, the introduction of a booster dose in countries could be justifiable.

The target age for SIAs is unclear in the WHO guidelines, as epidemiological characteristics vary by country (172). Based on the seroprevalence survey in an epidemic-prone area, we recommend that SIA should target 1–17 years (children and young adolescents) in Vietnam or countries with similar epidemiological backgrounds. The target area and age should be carefully planned according to the available resources and epidemiological priority. Furthermore, it should also be noted that transmission of diphtheria is not stopped immediately by SIAs.

Diphtheria easily resurges when a certain proportion of the population becomes susceptible. Of note, multiple large outbreaks occurred in refugee camps and unstable societies in the late 2010s and early 2020s, where children's routine immunisation programmes were disrupted (49, 55, 86). Diphtheria cases can be identified in well-vaccinated communities if individuals remain susceptible (115). For example, an unvaccinated child was diagnosed with diphtheria in a community with high vaccination coverage Spain in 2014 without any travel history or contact with other diphtheria cases (251).

The research in this thesis confirmed that classical toxigenic *C. diphtheriae* was the causal pathogen of the recent outbreak in Vietnam. The transmission pattern and pathogenicity of *C. diphtheriae* has not changed since the pre-vaccination era. Primary transmission occurs among close contacts, including members of the same household or peers in school dormitories. Transmission initially appears to be contained in small areas but gradually expands to neighbouring areas. Transmission most likely continues through either vaccinated or unvaccinated asymptomatic carriers. Some asymptomatic carriers harbour a non-toxigenic strain, while others harbour a toxigenic strain. Future MLST of identified cases and carriers among members of the same household and community will provide evidence of lysogenic conversion in the host during an epidemic.

It should be noted that no large diphtheria outbreaks were reported where high infant vaccination coverage was maintained even without school-entry or adult booster doses (i.e., in Nha Trang city, Vietnam). Therefore, it is speculated that a large outbreak would not occur if the susceptible individuals are limited to adults. One potential explanation is that child asymptomatic carriers may have biological factors for accelerating the lysogenic conversion of corynephage β in *C. diphtheriae*, such as low serum iron concentration. Another explanation is, according to the current demographic characteristics of epidemic-prone areas, children account for a high proportion of the population. For example, children aged 5 years or younger accounted for 10% of the study population in Vietnam in 2019 (209), which

should be higher in other settings. Therefore, the same proportion of susceptible children affects the entire population more significantly than adults. Alternatively, adults might have acquired cellular immunity from natural infection over the course of their life, although ELISA may not detect the antibody in their serum. In either case, eradicating the toxigenic strain from children is important for effectively eliminating the toxigenic strain in entire communities. Identifying the favourable host environment for corynephage to convert to a lysogenic form may be one study area to help understand the transmission of *Corynebacterium* species. Future development of new vaccines with universal surface proteins of *Corynebacterium* species may be more effective in reducing carriage and controlling diphtheria.

Several large outbreaks have been observed in the last decade, including in Bangladesh, Yemen, and Venezuela. The common features among these outbreaks were low infant vaccination coverage, large-scale population movement, and crowded housing in refugee camps; they were common risk factors for large-scale diphtheria outbreaks. In addition, this research clearly demonstrated that the protection level against diphtheria in a vaccinated community, especially a well-vaccinated community is low, except for preschool children. If the infant vaccination programme is halted for 1 year leading to the loss of a 1-year population with the highest protection level, it is projected that the protection level of the entire population will drop significantly. The findings of this study indicate that a sudden decline in infant vaccination coverage triggers diphtheria outbreaks.

Southeast Asian countries have experienced several outbreaks since the 2010s. Each country has had different levels of vaccination coverage for the last decades, while DTP was introduced at a similar time when the EPI was initiated. The transmission pattern of *Corynebacterium* species and the waning pattern of vaccine-derived anti-diphtheria toxoid antibodies could be similar between Vietnam and other Southeast Asian countries. Additional studies will be necessary to understand the epidemiology of diphtheria in each country. In general, vaccination programmes in Southeast Asian countries have not been sufficient to eliminate toxigenic strains of *C. diphtheriae.* Further efforts to increase vaccination coverage, thereby increasing the proportion of protected individuals, are essential to controlling diphtheria in this region.

The main research questions of this thesis were: ‘Is it required to introduce a school-entry booster dose as a national vaccination strategy in Vietnam? If so, which age is appropriate? Is the school-entry booster dose effective to stop the ongoing outbreak?’ The answer to these questions is to identify the appropriate vaccination policy for diphtheria control in Vietnam. We found that the seroprevalence in school-age children was markedly low, and

anti-diphtheria toxoid antibodies derived from three or four doses of DTP waned rapidly in the first 5 years after vaccination in Vietnam. This is a unique finding in the research area of diphtheria serology. Therefore, increased uptake of the primary dose series of DTP and the introduction of a school-entry booster dose is recommended to control diphtheria in Vietnam. Adequate coverage of primary and booster doses must be maintained for several decades to reduce the prevalence of toxigenic strains of *C. diphtheriae.*

This thesis identified that the vaccine-derived immunity wanes quickly within a decade after the last vaccination, therefore, multiple booster doses are necessarily to protect all age groups. Unless the all age groups including older adults are protected by booster doses, the toxigenic *C. diphtheriae* may not be eliminated. Booster doses protecting the older population will also be required in the future.

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Appendices

Appendix 1: Chapter 4- Search strategy and results in three database

All the electric search in each database was conducted on March 3, 2020.

Database: Ovid MEDLINE(R) and In-Process & Other Non-Indexed Citations and Daily <1946 to March 03, 2020> Search Strategy:

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1 exp Corynebacterium/ or exp diphtheria/ (15098)
2 (corynebacteirum or diphtheria*).mp. (21841)
3 1 or 2 (28637)
4 exp vaccines/ or exp diphtheria toxoid/ or exp Immunization/ or exp vaccination/ (316111)
5 (vaccine* or diphtheria toxoid or immuni#ation or schedule or vaccination).mp. (588335)
6 4 or 5 (606348)
7 seroepidemiologic studies/ or serology/ (20974)
8 (seroepidemiolog* or seroprevalence or serology or serological survey or (immune adj3 status)).mp. (64225)
9 7 or 8 (64225)
10 3 and 6 and 9 (295)

Database: Embase Classic+Embase <1947 to 2020 March 03> Search Strategy:

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1 exp Corynebacterium/ or exp diphhteria/ (16393)
2 (Corynebacterium or diphtheria*).mp. (51887)
3 1 or 2 (51894)
4 exp vaccine/ or exp immunization/ or exp diphtheria toxoid/ or exp vaccination/ (498589)
5 (vaccine* or immuni#ation or schedule or diphtheria toxoid or vaccination).mp. (681904)
6 4 or 5 (697074)
7 exp seroprevalence/ or exp seroepidemiology/ or exp immune status/ or serology/ (107502)
8 (seroepidemiolog* or seroprevalence or serological survey or (immune adj3 status)).mp. (50987)
9 7 or 8 (126225)
10 3 and 6 and 9 (701)

Database: Global Health <1910 to 2020 Week 08> Search Strategy:

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1 exp corynebacterium/ or exp diphtheria/ (12090)
2 (corynebacterium or diphtheria*).mp. (14805)
3 1 or 2 (14811)
4 exp vaccines/ or exp immunization/ or exp vaccination/ or exp diphtheria toxoids/ (128387)
5 (vaccine* or immuni#ation or schedule or vaccination or diphtheria toxoid*).mp. (170384)
6 4 or 5 (170400)
7 exp serological surveys/ or exp seroprevalence/ or exp serology/ (37749)
8 (seroepidemiolog* or seroprevalence or (immune adj3 status)).mp. (34520)
9 7 or 8 (44097)
10 3 and 6 and 9 (208)

Appendix 2: Chapter 4- Data extraction sheet

author		
country		
study_year		
studyend_year		
serological assay		
age_group		
sample size n		
positive (>=1.0) n		
positive (>=0.1) n		
positive (>=0.01) n		
study_design		
population (community or facility)		
sampling (random or prospective)		
response rate		
Comment on study design		
primary dose schedule		
boost1 (1-4y)		
boost2 (4-7y)		
boost3 (9-14y)		
boost4		
every10 years booster (Yes or No)		
coverage_primary (%)		
coverage_booster (%)		
year DTP introduced		
year booster dose introduced		

*GMC was extracted by WebPlotDigitizer

Appendix 3: Chapter 4- Critical appraisal (Hoy's criteria for prevalence study)

List of 10 questions (Q1 – 10) applied to the studies: YES=1 NO=0

1. Was the study's target population a close representation of the national population in relation to relevant variables, e.g., age, sex, occupation?
2. Was the sampling frame a true or close representation of the target population?
3. Was some form of random selection used to select the sample, OR, was a census undertaken?
4. Was the likelihood of non-response bias minimal?
5. Were data collected directly from the subjects (as opposed to a proxy)?
6. Was an acceptable case definition used in the study?
7. Was the study instrument that measured the parameter of interest shown to have reliability and validity (if necessary)?
8. Was the same mode of data collection used for all subjects?
9. Was the length of the shortest prevalence period for the parameter of interest appropriate?
10. Were the numerator(s) and denominator(s) for the parameter of interest appropriate?

Data extraction sheet for critical appraisal

Score>8 : low bias, score 6-8: medium bias, score <6: high bias

Reference	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Overall score
example	1	1	0	1	1	1	1	1	1	1	9

Appendix 4: Chapter 4- PRISMA Check list

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	

Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	

Appendix 5: Chapter 4- PROSPERO approval

PROSPERO
International prospective register of systematic reviews

NHS National Institute for Health Research

UNIVERSITY of York
Centre for Reviews and Dissemination

Systematic review

1. * Review title.

Give the working title of the review, for example the one used for obtaining funding. Ideally the title should state succinctly the interventions or exposures being reviewed and the associated health or social problems. Where appropriate, the title should use the PI(E)COS structure to contain information on the Participants, Intervention (or Exposure) and Comparison groups, the Outcomes to be measured and Study designs to be included.

Seroprevelence of diphtheria antitoxin antibody by different national immunization schedule, among preschool, school age children and adults: A Systematic review and a meta-analysis

2. Original language title.

For reviews in languages other than English, this field should be used to enter the title in the language of the review. This will be displayed together with the English language title.

Seroprevelence of diphtheria antitoxin antibody by different national immunization schedule, among preschool, school age children and adults: A Systematic review and a meta-analysis

3. * Anticipated or actual start date.

Give the date when the systematic review commenced, or is expected to commence.
01/04/2020

4. * Anticipated completion date.

Give the date by which the review is expected to be completed.
30/09/2020

5. * Stage of review at time of this submission.

Indicate the stage of progress of the review by ticking the relevant Started and Completed boxes. Additional information may be added in the free text box provided.
Please note: Reviews that have progressed beyond the point of completing data extraction at the time of initial registration are not eligible for inclusion in PROSPERO. Should evidence of incorrect status and/or completion date being supplied at the time of submission come to light, the content of the PROSPERO record will be removed leaving only the title and named contact details and a statement that inaccuracies in the stage of the review date had been identified.
This field should be updated when any amendments are made to a published record and on completion and publication of the review. If this field was pre-populated from the initial screening questions then you are not able to edit it until the record is published.

The review has not yet started: No

Review stage	Started	Completed
Preliminary searches	Yes	No
Piloting of the study selection process	Yes	No
Formal screening of search results against eligibility criteria	Yes	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

Provide any other relevant information about the stage of the review here (e.g. Funded proposal, protocol not yet finalised).

protocol may be modified in the due course of review process

protocol may be modified in the due course of review process

6. * Named contact.

The named contact acts as the guarantor for the accuracy of the information presented in the register record.

Noriko Kitamura

Email salutation (e.g. "Dr Smith" or "Joanne") for correspondence:

Dr Kitamura

7. * Named contact email.

Give the electronic mail address of the named contact.

noriko.kitamura@lshtm.ac.uk

8. Named contact address

Give the full postal address for the named contact.

Keppel street, WC1E 7HT, London, UK

9. Named contact phone number.

Give the telephone number for the named contact, including international dialling code.

+447453521185

10. * Organisational affiliation of the review.

Full title of the organisational affiliations for this review and website address if available. This field may be completed as 'None' if the review is not affiliated to any organisation.

London School of Hygiene and Tropical Medicine

Organisation web address:

https://www.lshtm.ac.uk/

11. * Review team members and their organisational affiliations.

Give the personal details and the organisational affiliations of each member of the review team. Affiliation refers to groups or organisations to which review team members belong. **NOTE: email and country are now mandatory fields for each person.**

Dr Noriko Kitamura. London School of Hygiene and Tropical Medicine
Mr Elvis Chem. London School of Hygiene and Tropical Medicine
Ms Khawater Bahkali. London School of Hygiene and Tropical Medicine
Ms Nayantara Wijayanandara. London School of Hygiene and Tropical Medicine

12. * Funding sources/sponsors.

Give details of the individuals, organizations, groups or other legal entities who take responsibility for initiating, managing, sponsoring and/or financing the review. Include any unique identification numbers assigned to the review by the individuals or bodies listed.

Noriko Kitamura initiated and manage this review. Noriko Kitamura designed the search strategy, determined the eligibility criteria and created data extraction sheet.

Noriko Kitamura received WISE scholarship from Ministry of Education, Science, Culture, Spots and Technology, Japan. Elvis Chem received schoalrship from Wellcome Trust, UK.

Either funding bodies was not involved in any decision or management of this review activities.

Grant number(s)

13. * Conflicts of interest.

List any conditions that could lead to actual or perceived undue influence on judgements concerning the main topic investigated in the review.

None

14. Collaborators.

Give the name and affiliation of any individuals or organisations who are working on the review but who are not listed as review team members. **NOTE: email and country are now mandatory fields for each person.**

15. * Review question.

State the question(s) to be addressed by the review, clearly and precisely. Review questions may be specific or broad. It may be appropriate to break very broad questions down into a series of related more specific questions. Questions may be framed or refined using PI(E)COS where relevant.

My main research question is to compare seroprevalence of diphtheria antitoxin antibody among healthy population by different booster vaccination schedule.

The specific objectives are to compare:

Seroprevalence among pre-school children with or without the booster dose given between 12 and 24 months of age Seroprevalence among school age children with or without the school-entry booster dose given between 4 and 7 years of age

Seroprevalence among adults older than 18 years old as a long-term immunity by ages of last booster dose,

4-7 years or 10-19 years of age.

16. * Searches.

State the sources that will be searched. Give the search dates, and any restrictions (e.g. language or publication period). Do NOT enter the full search strategy (it may be provided as a link or attachment.)

The electric database of MEDLINE, EMBASE, and Global Health were screened by using the following text and subject headings on each database on March 3, 2020. ("Corynebacterium" or "diphtheria") and ("vaccine*" or "vaccination" or "immuni#ation" or "schedule" or "diphtheria toxoid") and ("seroepidemiolog*" or "seroepidemiologic studies" or "seroprevalence" or "serology" or "serological survey" or "immune adj3 status"). Manual search was conducted by screening the reference list of the retrieved full-text articles.

At the stage of screening, no publication date, language or geographic restrictions were applied.

17. URL to search strategy.

Give a link to a published pdf/word document detailing either the search strategy or an example of a search strategy for a specific database if available (including the keywords that will be used in the search strategies), or upload your search strategy.Do NOT provide links to your search results.

MEDLINE subject heading is used in this example

1 exp Corynebacterium/

2 exp diphtheria/

3 corynebacteirum. mp.

4 diphtheria*.mp.

5 1 or 2 or 3 or 4

6 exp vaccines/

7 exp diphtheria toxoid/

8 exp Immunization/

9 exp vaccination/

10 vaccine* .mp.

11 diphtheria toxoid.mp. or

12 immuni#ation .mp.

13 schedule .mp.

14 vaccination.mp.

15 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14

16 seroepidemiologic studies/

17 serology/

18 seroepidemiolog* .mp.

19 seroprevalence .mp.

20 serology.mp.

21 serological survey.mp.

22 immune adj3 status.mp.

23 16 or 17 or 18 or 19 or 20 or 21 or 22

24 5 and 15 and 23

Alternatively, upload your search strategy to CRD in pdf format. Please note that by doing so you are consenting to the file being made publicly accessible.

Do not make this file publicly available until the review is complete

18. * Condition or domain being studied.

Give a short description of the disease, condition or healthcare domain being studied. This could include health and wellbeing outcomes.

Immune status measured by specified laboratory assay (e.g. ELISA or toxin neutralization assay) against diphtheria among healthy pre-school children, school age children and adults

19. * Participants/population.

Give summary criteria for the participants or populations being studied by the review. The preferred format includes details of both inclusion and exclusion criteria.

Inclusion criteria is if studies measured immunity among national or subnational population who supposed to receive diphtheria contained vaccine (e.g. DTP, DTP-Hib- HepB, DT, or TdaP) according to their national immunization program or among residual sera at facility (e.g. national/regional laboratory or hospitals) by laboratory assay of diphtheria antitoxin antibody. Immunogenicity study will not be eligible for this review, though the studies followed up immunity long term (more than one year after vaccination) may be included.

The studies assessed the immunity among immunocompromised host, such as patients infected HIV or other diseases, malignancy or post- organ transplant, etc. will not be included. The studies measured seroprevalence among migrants or refugees who did not follow the national immunization program in the countries where study was conducted will not be included.

The studies will be excluded if: (i) studies published not in full text (e.g. conference posters, abstracts), (ii) studies whose full texts were NOT written in English or Japanese, (iii) full text were not available after two library searches and contacted to the authors, and (iv) study did not stratify the age for seroprevalence, not provide laboratory method, use different cut-off value or not provide adequate information of population.

20. * Intervention(s), exposure(s).

Give full and clear descriptions or definitions of the nature of the interventions or the exposures to be reviewed.

Exposure is the booster dose of diphtheria contained vaccine (e.g. DTP) given at 1-2 years of age when we compare the immunity among pre-school and school-age children.

Exposure is the booster dose of diphtheria contained vaccine (e.g. DT, Td or TdaP) given at 1-2 years and/or 4-7 years of age when we compare the immunity among pre-school and school-age children.

Exposure is the booster dose of diphtheria contained vaccine (e.g. DT, Td or TdaP) given at 4-7 years and/or 9-14 years of age when we compare the immunity among adults.

21. * Comparator(s)/control.

Where relevant, give details of the alternatives against which the main subject/topic of the review will be compared (e.g. another intervention or a non-exposed control group). The preferred format includes details of both inclusion and exclusion criteria.

Schedules of DTP were categorized as four:

1) three primary dose with or without booster dose between 12 and 24 months (3p (+18m)),

2) three primary dose with or without a booster dose between 12 and 24 months and a booster dose between 4 and 7 years (3p (+ 18m) + 4-7y),

3) three primary dose with or without a booster dose between 12 and 24 months and a booster dose at 10 years (3p (+18m) + 10y), and

4) 3p + 18m and booster dose at 4-7 years and between 9 and 14 years (3p + 18m + 4-7y + 9-14y) which is the current WHO recommended schedule.

Seroprevalence of pre-school and school-age children with or without receiving booster dose between 12 and 24 months will be compared. In addition, seroprevalence of school-age children with or without 4-7 years old booster dose will be compared. The third comparison will be the seroprevalence of adults with different timing of last booster dose, 4-7 years or 10-14 years among groups 2), 3) and 4).

22. * Types of study to be included.

Give details of the types of study (study designs) eligible for inclusion in the review. If there are no restrictions on the types of study design eligible for inclusion, or certain study types are excluded, this should be stated. The preferred format includes details of both inclusion and exclusion criteria.

Cross sectional studies are eligible to be included in the review. Intervention studies (immunogenicity studies) or cohort studies following up more than one year after the vaccination will be included.

23. Context.

Give summary details of the setting and other relevant characteristics which help define the inclusion or exclusion criteria.

Research in low- and middle-income countries as well as high- income countries will be included.

For the analysis of adults immunity, only high-income countries which introduced vaccine more than 50 years ago at the time of study will be included as adults in low- and middle income countries have not been vaccinated according to the current national immunization program.

24. * Main outcome(s).

Give the pre-specified main (most important) outcomes of the review, including details of how the outcome is defined and measured and when these measurement are made, if these are part of the review inclusion criteria.

Main outcome is seroprevalence among pre-school children.

Seropositivity is measured by laboratory assay (e.g. ELISA or toxin neutralization assay) using the specified cut-off value. For ELISA, cut-off value 0.1 IU/ml is used for determining seropositive. For toxin neutralization assay, cut-off value 0.01IU/ml and 0.1IU/ml are used for determining seropositive. If other test was used, each paper's definition either 0.1 or 0.01IU/ml will be used. We conduct subgroup analysis for two main laboratory assay, ELISA and toxin neutralization assay.

* Measures of effect

Please specify the effect measure(s) for you main outcome(s) e.g. relative risks, odds ratios, risk difference, and/or 'number needed to treat.

Our effect measure is seroprevalence (proportion) among certain age categories. We need seropositive indivisual number (n) and tested number as a denominator (N) to calculate seroprevalence (proportion =n/N) for analysis.

25. * Additional outcome(s).

List the pre-specified additional outcomes of the review, with a similar level of detail to that required for main outcomes. Where there are no additional outcomes please state 'None' or 'Not applicable' as appropriate to the review

None

* Measures of effect

Please specify the effect measure(s) for you additional outcome(s) e.g. relative risks, odds ratios, risk difference, and/or 'number needed to treat.

None

26. * Data extraction (selection and coding).

Describe how studies will be selected for inclusion. State what data will be extracted or obtained. State how this will be done and recorded.

Two reviewers will independently screen all the potential studies obtained as results of search strategy from

titles and abstracts of the reference after de-duplication. Two reviewers independently assess selection of studies for inclusion in this review.We resolved discrepancies through discussion or, if required, we consult the third reviewer. We use EndNote for deduplication and sharing and recording the studies.

We create data extraction sheet by Microsoft Excel 2010. For included studies, two reviewers independently extract data from full-text articles. We resolve discrepancies through discussion or, if required, we consult the third reviewer. The following information will be extracted: study type, publication year, country, study year(s), sample size, sampling method, age(range and category), number of seropositive subjects, vaccination schedule and type, year of DTP introduction, method of serological assay, and cut-off values for assay.

If only total sample size and percentage of seropositive subjects are available, number of seropositive subjects will be calculated from sample size and percentage. If the full-text artice used graph to show the seroprevalence, we identify the numeric seroprevalence using digital software.
Diphtheria vaccination schedule and vaccine type used in the population, year of DTP introduction will be identified from each full-text article. If not available, the infomration will be found on the WHO portal website (http://apps.who.int/immunization_monitoring/globalsummary/) or other sources (e.g. studies by Galazka et al. Vaccine. 1996).

27. * Risk of bias (quality) assessment.

Describe the method of assessing risk of bias or quality assessment. State which characteristics of the studies will be assessed and any formal risk of bias tools that will be used.

Majority of the included studies will be cross-sectional studies. Assessment of risk of bias in individual studies will be carried out using Hoy's tool, which is developed by Hoy et.al. in 2012, for prevalence studies. Assessment of risk of bias of some cohort studies will be carried out using Newcastle Ottawa quality assessment tool.
Risk of bias in individual studies will be assessed by two investigators. Any disagreement were assessed by third investigator.

28. * Strategy for data synthesis.

Provide details of the planned synthesis including a rationale for the methods selected. This **must not be generic text** but should be **specific to your review** and describe how the proposed analysis will be applied to your data.

Narrative and quantitative summary table will be created by age categories.

Pooled estimate of seroprevalence in each age group will be measured by MetaXL version 5.3 with 95% CI. Results will be visualized in Forest plot by age categories and by different immunization schedule.

Publication bias will be assessed by Funnel plot, Doi plot, and LFK index using MetaXL.

Heterogeneity of data was assessed and Q statistics I^2, and $?^2$ will be reported.

Depending on the value of I^2, random-effect method will be applied.

29. * Analysis of subgroups or subsets.

State any planned investigation of 'subgroups'. Be clear and specific about which type of study or participant will be included in each group or covariate investigated. State the planned analytic approach.

Sub-group analysis will be conducted with our without specific booster dose vaccination in each age category.

Sub-group analysis will be conducted by method of serology assay, mainly ELISA and Neutralization assay (TNT), to assess the difference or consistency among laboratory assays.

30. * Type and method of review.

Select the type of review and the review method from the lists below. Select the health area(s) of interest for your review.

Type of review

Cost effectiveness
No

Diagnostic
No

Epidemiologic
Yes

Individual patient data (IPD) meta-analysis
No

Intervention
No

Meta-analysis
Yes

Methodology
No

Narrative synthesis
Yes

Network meta-analysis
No

Pre-clinical
No

Prevention
No

Prognostic
No

Prospective meta-analysis (PMA)
No

Review of reviews
No

Service delivery
No

Synthesis of qualitative studies
No

Systematic review
Yes

Other
No

Health area of the review

Alcohol/substance misuse/abuse
No

Blood and immune system
No

Cancer
No

Cardiovascular
No

Care of the elderly
No

Child health
Yes

Complementary therapies
No

Crime and justice
No

Dental
No

Digestive system
No

Ear, nose and throat
No

Education
No

Endocrine and metabolic disorders
No

Eye disorders
No

General interest
No

Genetics
No

Health inequalities/health equity
No

Infections and infestations
Yes

International development
No

Mental health and behavioural conditions
No

Musculoskeletal
No

Neurological
No

Nursing
No

Obstetrics and gynaecology
No

Oral health
No

Palliative care
No

Perioperative care
No

Physiotherapy
No

Pregnancy and childbirth
No

Public health (including social determinants of health)
No

Rehabilitation
No

Respiratory disorders
No

Service delivery
No

Skin disorders
No

Social care
No

Surgery
No

Tropical Medicine
No

Urological
No

Wounds, injuries and accidents
No

Violence and abuse
No

31. Language.

Select each language individually to add it to the list below, use the bin icon to remove any added in error.
English

There is an English language summary.

32. * Country.

Select the country in which the review is being carried out from the drop down list. For multi-national collaborations select all the countries involved.
England

33. Other registration details.

Give the name of any organisation where the systematic review title or protocol is registered (such as with The Campbell Collaboration, or The Joanna Briggs Institute) together with any unique identification number assigned. (N.B. Registration details for Cochrane protocols will be automatically entered). If extracted data will be stored and made available through a repository such as the Systematic Review Data Repository (SRDR), details and a link should be included here. If none, leave blank.

34. Reference and/or URL for published protocol.

Give the citation and link for the published protocol, if there is one

Give the link to the published protocol.

Alternatively, upload your published protocol to CRD in pdf format. Please note that by doing so you are consenting to the file being made publicly accessible.

No I do not make this file publicly available until the review is complete

Please note that the information required in the PROSPERO registration form must be completed in full even if access to a protocol is given.

35. Dissemination plans.

Give brief details of plans for communicating essential messages from the review to the appropriate audiences.

The results of this review will be submitted to a peer-review journal in this field. The results of this review will provide the evidence for the effective booster dose schedule for low-income countries which need to introduce booster dose in the future.

Do you intend to publish the review on completion?

Yes

36. Keywords.

Give words or phrases that best describe the review. Separate keywords with a semicolon or new line. Keywords will help users find the review in the Register (the words do not appear in the public record but are included in searches). Be as specific and precise as possible. Avoid acronyms and abbreviations unless these are in wide use.

Systetmatic review; meta-analysis; diphtheria; serprevalence; immunity; pre-school child; school-age child; adults

37. Details of any existing review of the same topic by the same authors.

Give details of earlier versions of the systematic review if an update of an existing review is being registered, including full bibliographic reference if possible.

38. * Current review status.

Review status should be updated when the review is completed and when it is published. For newregistrations the review must be Ongoing.
Please provide anticipated publication date

Review_Ongoing

39. Any additional information.

Provide any other information the review team feel is relevant to the registration of the review.

40. Details of final report/publication(s).

This field should be left empty until details of the completed review are available.

Give the link to the published review.

Appendix 6: Chapter 5 and 6- Ethics approvals

Appendix 6-1. Ethics approval from LSHTM

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Dr Noriko Kitamura
LSHTM

10 July 2019

Dear Noriko ,

Study Title: Diphtheria seroprevalence survey in Nha Trang, Vietnam

LSHTM ethics ref: 17518

Thank you for your application for the above research, which has now been considered by the Observational Committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Local Approval	VN01057-272015-3rdPhase-Dengue-approval	14/09/2015	1
Local Approval	VN01057-282015-3rdPhase-ARI-approval	14/09/2015	1
Investigator CV	CV_NorikoKitamura	01/05/2019	1
Investigator CV	Emilia Vynnycky - CV	01/05/2019	1
Investigator CV	Michiko ToizumI - CV	01/05/2019	1
Investigator CV	YoshidaLayMyint - CV	01/05/2019	1
Investigator CV	CV Dr DUC ANH	01/05/2019	1
Consent form	English_ICF for community dengue-NP study	08/05/2019	1
Protocol / Proposal	Cover letter	30/05/2019	1
Protocol / Proposal	Protocol for LEO	31/05/2019	1

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

[redacted]

Professor Jimmy Whitworth

Chair

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Dr Noriko Kitamura
LSHTM

6 July 2020

Dear Noriko

Study Title: Diphtheria carriage and seroprevalence among population in outbreak setting and waning of vaccine (DTP) derived immunity in Vietnam

LSHTM Ethics Ref: 17913

Thank you for responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Investigator CV	CV_NorikoKitamura	11/11/2019	1
Investigator CV	Emilia Vynnycky - CV	11/11/2019	1
Investigator CV	Michiko ToizumI - CV	11/11/2019	1
Investigator CV	YoshidaLayMyint - CV	11/11/2019	1
Protocol / Proposal	protocol diphtheria carriage prevalence and seroprevalence survey20190912	12/11/2019	1
Protocol / Proposal	Data collection form_20190919_EN	12/11/2019	1
Consent form	ICF_EN_NhaTrang2017and2019	14/11/2019	1
Consent form	ICF_EN_QuangNgai2019	21/11/2019	1
Local Approval	PasteurNT-diphtheria-ethical approval-2019	13/04/2020	1
Local Approval	VN01057-272015-3rdPhase-Dengue-approval	13/04/2020	1
Local Approval	VN01057-282015-3rdPhase-ARI-approval	13/04/2020	1
Local Approval	17518LSHTMapproval20190710	13/04/2020	1
Investigator CV	Paul_Fine-CV2	17/04/2020	1
Investigator CV	CV- Hung Thai Do	01/07/2020	1
Investigator CV	CV-Thanh	06/07/2020	1
Investigator CV	CV-LIEN-PINT	06/07/2020	1
Covering Letter	Cover Letter	06/07/2020	1
Covering Letter	Pasteur NT pathogen survey-study-contract 2019Sept-both signed	06/07/2020	1

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

[redacted]

Professor Jimmy Whitworth
Chair

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Improving health worldwide

Appendix 6-2. Ethics approval from MoH Vietnam

BỘ Y TẾ
HỘI ĐỒNG ĐẠO ĐỨC TRONG NCYSH
VIỆN PASTEUR NHA TRANG

Số 04 /IPN-HĐĐĐ

CỘNG HÒA XÃ HỘI CHỦ NGHĨA VIỆT NAM
Độc lập – Tự do – Hạnh phúc

Khánh Hòa, ngày 15 tháng 09 năm 2019

CHẤP THUẬN (CHO PHÉP) CỦA HỘI ĐỒNG ĐẠO ĐỨC TRONG NGHIÊN CỨU Y SINH HỌC VIỆN PASTEUR NHA TRANG

Căn cứ Quyết định số 356/QĐ-IPN ngày 01 tháng 03 năm 2019 của Phó Viện trưởng phụ trách Viện Pasteur Nha Trang về việc kiện toàn Hội đồng đạo đức trong nghiên cứu y sinh học Viện Pasteur Nha Trang;

Căn cứ Biên bản họp xét duyệt ngày 13 tháng 9 năm 2019 của Hội đồng đạo đức trong nghiên cứu y sinh học Viện Pasteur Nha Trang;

Hội đồng đạo đức trong nghiên cứu y sinh học Viện Pasteur Nha Trang **chấp thuận (cho phép)** về các khía cạnh đạo đức đối với nghiên cứu ***"Điều tra huyết thanh học thực trạng tồn lưu kháng thể và tỷ lệ nhiễm bạch hầu tại 2 huyện miền núi Tây Trà và Sơn Hà, tỉnh Quảng Ngãi, Việt Nam"***

- Mã số: **04/2019/HĐĐĐ-IPN**
- Quy trình xét duyệt: nghiên cứu được xem xét theo quy trình đầy đủ.
- Đơn vị triển khai nghiên cứu: Khoa Dịch tễ, Khoa Vi khuẩn, Viện Pasteur Nha Trang
- Cơ quan chủ quản: Viện Y học nhiệt đới, Đại học tổng hợp Nagasaki, Nhật Bản
- Đồng chủ nhiệm đề tài: TS.BS. Đỗ Thái Hùng –Phó Viện trưởng phụ trách
- Địa điểm tiến hành nghiên cứu: tại 2 huyện miền núi Tây Trà và Sơn Tây của tỉnh Quảng Ngãi.
- Thời gian tiến hành nghiên cứu:
  - Đợt 1: 10-11/2019 và Đợt 2: 11/2020
  - Tại Nhật Bản: 12/2019- 12/2021

Các yêu cầu cụ thể của Hội đồng đạo đức trong nghiên cứu y sinh học Viện Pasteur Nha Trang:

- Tuân thủ theo biên bản xét duyệt của Hội đồng trong suốt quá trình triển khai đề tài. Báo cáo Hội đồng nếu có vấn đề phát sinh trong quá trình triển khai đề tài.
- Dừng nghiên cứu hoặc bổ sung, sửa đổi các nội dung của đề tài nếu nhận thấy có nhiều nguy cơ cho đối tượng nghiên cứu, người tham gia nghiên cứu.
- Thường xuyên báo cáo cho Hội đồng về tiến trình thực hiện đề tài. Báo cáo Hội đồng để được phê duyệt nếu có bất kỳ sự thay đổi nào về phương pháp nghiên cứu, nội dung nghiên cứu, đối tượng nghiên cứu.
- ***Thời gian được chấp thuận (cho phép):*** Từ 10/2019- 11/2020
  Lưu ý: Hội đồng đạo đức có thể kiểm tra ngẫu nhiên trong thời gian tiến hành nghiên cứu.

THƯ KÝ

[redacted]

Nguyễn Thành Đông

CHỦ TỊCH

[redacted]

Trịnh Thị Bích Thủy

Nơi nhận:
- Chủ nhiệm đề tài;
- Khoa Dịch tễ;
- Lưu: HĐĐĐ.

XÁC NHẬN CỦA
VIỆN PASTEUR NHA TRANG
KT. VIỆN TRƯỞNG
PHÓ VIỆN TRƯỞNG

[redacted]

Lê Xuân Huy

Appendix 6-3. Ethics Approval from Quang Ngai province, Vietnam

BỘ Y TẾ
VIỆN PASTEUR NHA TRANG

Số: 1775/IPN-DT
V/v xin chấp thuận điều tra đánh giá huyết thanh học và tỷ lệ hiện nhiễm bạch hầu trong cộng đồng

CỘNG HÒA XÃ HỘI CHỦ NGHĨA VIỆT NAM
Độc lập- Tự do- Hạnh phúc

Khánh Hòa, ngày 04 tháng 9 năm 2019

Kính gửi: Sở Y tế tỉnh Quảng Ngãi

Nhằm mục đích đánh giá sự tồn lưu kháng thể kháng bạch hầu và tỷ lệ hiện nhiễm bạch hầu trong cộng đồng làm cơ sở cho việc triển khai công tác phòng chống dịch một cách hiệu quả. Được sự đồng ý của Bộ Y tế, trong khuôn khổ hợp tác với trường Đại học Nagasaki - Nhật Bản. Viện Pasteur Nha Trang dự kiến triển khai nghiên cứu "Điều tra huyết thanh học và tỷ lệ hiện nhiễm bạch hầu trong bối cảnh bùng phát dịch tại Quảng Ngãi, Việt Nam". Cụ thể như sau:

- ✓ Địa điểm: 02 huyện Sơn Hà và Tây Trà.
- ✓ Đối tượng: Người dân từ 1- 55 tuổi sinh sống trên địa bàn 02 huyện nêu trên.
- ✓ Thời gian:
  - Đợt 1: Tháng 9 – 10/2019
  - Đợt 2: Tháng 11/2020.

Để hoạt động nêu trên được triển khai thực hiện, Viện Pasteur Nha Trang xin ý kiến chấp thuận tham gia điều tra của Sở Y tế để chủ động trong công tác chuẩn bị, lập kế hoạch và tổ chức điều tra.

Công văn phản hồi xin gửi về Viện Pasteur Nha Trang trước ngày 12/9/2019. Chi tiết liên hệ: ThS.Hoàng Tiến Thanh- Trưởng khoa Dịch tễ,Viện Pasteur Nha Trang, số điện thoại: 0905.106627 hoặc BS. Đào Thế Anh, số điện thoại: 0914.481086.

Rất mong sớm nhận được công văn chấp thuận từ Sở Y tế.

Xin trân trọng cảm ơn./.

Nơi nhận:
- Như trên;
- Ban Giámđốc;
- Lưu: VT, DT.

PHÓ VIỆN TRƯỞNG PHỤ TRÁCH

[redacted]

Đỗ Thái Hùng

Appendix 6-4. Ethics Approval from Pasteur Institute in Nha Trang

BỘ Y TẾ
CỤC KHOA HỌC CÔNG NGHỆ VÀ ĐÀO TẠO

Số: 1046 /K2ĐT-KHCN
V/v triển khai nghiên cứu hợp tác

CỘNG HOÀ XÃ HỘI CHỦ NGHĨA VIỆT NAM
Độc lập - Tự do - Hạnh phúc

Hà Nội, ngày 23 tháng 10 năm 2019

Kính gửi: Viện Pasteur Nha Trang

Phúc đáp công văn số 1920/IPN-ĐT ngày 20 tháng 9 năm 2019 của Viện Pasteur Nha Trang về triển khai nghiên cứu hợp tác; Cục Khoa học công nghệ và Đào tạo có ý kiến như sau:

- Đồng thuận về chủ trương đối với nội dung khoa học của nghiên cứu: "Điều tra thực trạng tồn lưu kháng thể và tỷ lệ nhiễm Bạch hầu tại 2 huyện miền núi Tây Trà và Sơn Hà, tỉnh Quảng Ngãi, Việt Nam" nhằm cung cấp bằng chứng khoa học về tỷ lệ nhiễm bạch hầu không triệu chứng trong cộng đồng tại tỉnh Quảng Ngãi.

- Đề nghị Chủ nhiệm và Cơ quan chủ trì đề tài thực hiện đầy đủ, đúng các quy định hiện hành về hoạt động khoa học và công nghệ, đạo đức trong nghiên cứu y sinh học và hợp tác quốc tế trong lĩnh vực y tế.

Cục Khoa học công nghệ và Đào tạo thông báo đến đơn vị biết và thực hiện./.

Nơi nhận:
- Như trên;
- Thứ trưởng Nguyễn Trường Sơn (để b/c);
- Phó Cục trưởng Phụ trách (để b/c);
- Vụ HTQT (để phối hợp chỉ đạo);
- Lưu: VT, KHCN.

KT. CỤC TRƯỞNG
PHÓ CỤC TRƯỞNG

[redacted]

Nguyễn Minh Lợi

Appendix 7: Chapter 6- Standard operation protocol: Bacterial culture

Receipt of the samples at the lab:

- Check the label and sample collection sheet.
- Once swabs are received at the laboratory, all throat swabs should be kept at -30 degrees immediately until culture is conducted (until Tellurite medium is available). All STGG samples should be kept at -80 degrees.
- Culture should be completed within three months after the sample collection.

Appendix 7-1. Culture from Throat swab

1. After receiving the samples from the field, store throat swabs in a -30 degrees freezer until Tellurite medium is available.
2. Tellurite medium is prepared (600 plates).
3. One plate of medium is used for two samples. A line should be drawn in the middle of the plates to divide the plates by two.
4. Put the swab on the rim of the plate and put it back into the transport media.
5. Use a 10ul loop to spread the drop from the swab.
6. Incubate plates in a 35 degrees incubator for 24 hours. If the colony grows, move to step6. If not, incubate for another 24 hours (48 hours total).
7. Conduct gram stain of black colonies.
8. If the result of Gram stain is positive bacillus, transfer colonies to Sheep Blood Agar(SBA) medium, incubate plate in a 35 -37 degrees incubator for 24 hours.
9. Store colonies in BHI+ 20% glycerol tube at a -80-degree freezer.
10. After culture, put the throat swab into 1ml STGG, break the shaft, and close the lid. Vortex STGG.
11. Aliquot 200ul in Eppendorf tubes for DNA extraction.
12. Store STGG with the original throat swab at -80 degrees and store Eppendorf tubes in a -30-degree freezer.

Appendix 7-2. Culture from STGG media (NP swab)

1. After receiving the samples from the field, samples should be stored in – an 80-degree freezer. From original STGG tubes, aliquot 200ul for DNA extraction and 400ul for future culture.
2. Before freezing STGG again, we conduct a culture for C. diphtheria.
3. From 400ul tubes, using a 10 ul loop, inoculate STGG on the plates.
4. Incubate plates in a 35 degrees incubator for 24 hours. If the colony grows, move to step6. If not, incubate for another 24 hours (48 hours total).
5. Conduct gram stain of black colonies.

6. If the result of Gram stain is positive bacillus, transfer colonies to Sheep Blood Agar(SBA) medium, incubate plate in a 35-37 degrees incubator for 24 hours.
7. Store colonies in BHI+ 20% glycerol tube in a -80-degree freezer.

Appendix 8: Chapter 6- Standard operation protocol: real-time PCR

Appendix 8-1. DNA extraction from STGG media (Throat swab/NP swab)

1. Thaw previously aliquotted 200ul of STGG.
2. Vortex for 5 minutes
3. Centrifuge 16,000 x g for 5 minutes
4. After removal of supernatant, cell pellet was suspended in 180 uL of Tris-EDTA buffer (10mM Tris-HCl, pH 8.0 + 1mM EDTA).
5. Add 5 ul of lysozyme (100mg/ml) on suspension.
6. Incubate at 37℃ for 30 minutes.
7. Add 25uL of Qiagen Proteinase K.
8. Add 200uL of Buffer AL (Qiagen).
9. Vortex and incubate at 70℃ for 2 hours and then at 95℃ for 30 min.
10. Briefly centrifuge the microcentrifuge tube to remove drops inside the lid.
11. Add 200uL ethanol (96%) to the sample and mix by pulse-vortexing for 15 seconds.
12. Briefly centrifuge the microcentrifuge tube to remove drops inside the lid.
13. Carefully apply the mixture from step 6 to the QIAamp Spin column (in a 2 ml collection tube) without wetting the rim, close the cap, and centrifuge at 6000xg (8000rpm) for 1 min. Place the QIAamp Spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
14. Carefully open the QIAamp Spin column and add 500uL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000xg (8000rpm) for 1 min. Place the QIAamp Spin Column in a clean 2ml collection tube(provided), and discard the collection tube containing the filtrate.
15. Carefully open the QIAamp spin Colum and add 500uL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000xg, 14,000 rpm) for 3 min.
16. Discard the filtrate. And Place the QIAamp Spin column in the same tube. Centrifuge at 20,000 x g (14,000 rpm) for 1 min.
17. Place the QIAamp Spin Column in a clean 1.5ml microcentrifuge tube and discard the collection tube containing the filtrate.
18. Carefully open the QIAamp Spin Column and add 100uL Buffer AE. Incubate at room temperature (15-25℃) for 1min. Centrifuge at 6,000xg (8,000 rpm) for 1 minutes.

Appendix 8-2. qPCR for detection of *tox* gene and *Corynebacterium* species from DNA extracted from swabs

Materials

- Positive controls (DNA extracted from toxigenic C. diphtheriae and C. ulcerans. IPC: The IPC DNA comprises the pGFP plasmid, which contains the gfp gene (from Aequorea victoria) cloned into a bacterial plasmid. 10µL aliquots of 500 copies/µL stock are prepared and stored at -20°C or below. You can use gfp DNA cloned into a plasmid or any internal commercial control suitable for real-time PCR. If possible, prepare (aliquots of 500 copies/µL stock as 10x final concentration) and store (at -20°C in the PCR clean room freezer) in advance. Add internal control during the extraction.
- Nuclease-free water.
- 0.2mL real-time PCR tubes.
- 1.5mL sterile tubes.
- TE buffer

Primers and probes

Target gene	Oligo	Sequence	Fragment
C. diphtheria rpoB	dip_rpobF	CGTTCGCAAAGATTACGGAACCA	97bp
	dip_rpobR	CACTCAGGCGTACCAATCAAC	
	Cdip HP	Cy5-AGGTTCCGGGGCTTCTCGATATTCA-BHQ1	
C. ulcerans rpoB	ulc_rpobF	TTCGCATGGCTCATTGGCAC	98bp
	ulc_rpobR	TCCAGGATGTCTTCCAGTCC	
	CulcHP	Texas Red -CCAGCAGGAGGAGCTGGGTGAA-BHQ1	
tox	toxAF	CTTTTCTTCGTACCACGGGACTAA	117bp
	toxAR	CTATAAAACCCTTTCCAATCATCGTC	
	diptoxHP	HEX-AAGGTATACAAAAGCCAAAATCTGGTACACAAGG-BHQ2	
gfp	gfp_FP	CCTGTCCTTTTACCAGACAACCA	77bp
	gfp_RP	GGTCTCTCTTTTCGTTGGGATCT	
	gfp_HP	FAM-TACCTGTCCACACAATCTGCCCTTTCG-BHQ2	

Preparation of PCR mix for multiplex

I. Prepare the primers/probes mix in advance:

1. Mix 100µM (100pmol/µl) primers (forward and reverse) and probes per each target.

2. Prepare the mixture, including all real-time PCR primers and probe targets following the proportions described in the table below
3. Label the mixture as “Dip4plex”, indicating the final volume on the tube.
4. Before using each new batch of primer/probe mixture to test samples, perform a QC run using the positive control samples plus ≥1 negative control (non-template control, NTC).
5. The “Dip4plex” tube must be stored in a clean laboratory in a freezer at -20°C.

REAGENT		FOR 1ML OF MIX	FOR 1.5MLOF MIX	FOR 2MLOF MIX	FINAL CONC. IN 20X MIX
PRIMER/PROBE [100PMOL/µL STOCK]	dip rpob-F	50µl	75µl	100µl	5µM
	dip rpob-R	50µl	75µl	100µl	5µM
	C-dip HP	20µl	30µl	40µl	2µM
	ulc rpob-F	50µl	75µl	100µl	5µM
	ulc rpob-R	50µl	75µl	100µl	5µM
	C-ulc HP	20µl	30µl	40µl	2µM
	toxA-F	50µl	75µl	100µl	5µM
	toxA-R	50µl	75µl	100µl	5µM
	Diptox HP	20µl	30µl	40µl	2µM
	gfp-FP	50µl	75µl	100µl	5µM
	gfp-RP	50µl	75µl	100µl	5µM
	gfp HP	20µl	30µl	40µl	2µM
BUFFER	*TE 1x pH 8.0*	*520*µl	*780*µl	*1040*µl	
FINAL VOLUME		1000µl	1500µl		

1. In the PCR Clean Room, prepare the real time PCR reaction mix in a 1.5ml tube as described in the table below (and in the worksheet).

Reagent	qPCR mix x1 (µl)	qPCR mix x15 (µl)
PCR grade H2O	1µl	15µl
Dip4plex 20x	4µl	60µl
pGFP [50copies µl]	3 µl	45µl
Real time PCR master mix*	12 µl	180µl
Dispense 20µl in each tube		
Add 5µl of DNA Template		

2. Gently vortex the 1.5 centrifuge mL tube before dispensing **20 µl** of the reaction mix into 200 µL qPCR tubes.
3. In another cabinet, add to each tube **5µl** of DNA template previously extracted and the tubes for a few seconds.
4. Set up the real-time machine, running the saved program that would apply the cycles and the temperature described in the table below.

Cycling conditions:	Temperature:	Time per cycle:
PCR initial activation step	95ºC	2minutes
Denaturation	95°C	10seconds
Annealing/Extension	60ºC	45seconds

5. After the qPCR run has been completed, click the 'Analysis' button, and the software will display the Ct value of samples where the fluorescence crosses the threshold line.
6. Check the threshold in order to avoid a potential false negative, especially when the background fluorescence of the negative control samples rises slightly. If this happens, you may raise the threshold above 0.05 to prevent false Ct values. If, however, you need to raise the threshold above 0.1, discuss the results with a senior member of staff to assess whether the run needs to be repeated.

ll. Prepare *C.diphtheriae* and *C.ulcerans* control Standard curve from 1,000,000 copy/ul

1. Use 1,000,000 copy/ul stock
2. Prepare 1.5ml Eppendorf tube. Add 990 ul TE buffer, and add 10ul of control. (10,000 copy) store on the -20 for the next run.
3. Prepare 1.5ml Eppendorf tube. Take 40 ul of this and add it to the 960 ul TE buffer. This creates 400 copies/ul stock. Store on the -20 for the next run.
4. Then serially dilute by two times. Prepare 8 eppendorf tubes. Label 400, 200, 100, 50, 25,12.5, 6.25, and 3.125. Add 20ul TE buffer to each tube.
5. Take 20ul from 400 copies/ul stock. And add on the next tube. This is 200 copies/ul.
6. Take 20ul from 200 copies/ul stock. And add on the next tube. This is 100 copies/ul.
7. Take 20ul from 100 copies/ul stock. And add on the next tube. This is 50 copies/ul.
8. Take 20ul from 50 copies/ul stock. And add on the next tube. This is 25 copies/ul.
9. Take 20ul from 25 copies/ul stock. And add on the next tube. This is 12.5 copies/ul.
10. Take 20ul from 12.5 copy/ul stock. And add on the next tube. This is 6.25 copies/ul.
11. Take 20ul from 6.25 copy/ul stock. And add on the next tube. This is 3.125 copies/ul.

12. Repeat the same procedure for *C.diphtheriae* and *C.ulcerans*.

For the test run, prepare double standard curves with two negative controls.

Once the test run was completed, we just used 50 and 25 copies/ul for the positive control for each run.

Interpretation of results

1. Check that the Ct values for the standards lie within the Min and Max (mean ± 2 standard deviations) shown below. If they don't, discuss the results with a senior member of staff (this could be indicative of probe degradation and loss of sensitivity).
2. Interpret the PCR results for the test samples according to the table below.
3. If the PCR result indicates that the isolate is a toxin gene bearing *Corynebacterium diphtheriae,* or a toxin gene bearing *C. ulcerans*/*C. pseudotuberculosis*, the result needs to be confirmed by the modified Elek test.
4. If the result is *Inhibitory* or *Equivocal*, consider whether to repeat the PCR (and possibly the DNA extraction).

C.diphtheriae detected	*C. ulcerans/ C.pseudotuberculosis* detected	Toxin gene detected	IPC amplified	Final result
+	-	-	+	Non-toxin gene bearing *C. diphtheriae* detected
+	-	+	+	Toxin gene bearing *C. diphtheriae* detected
-	+	-	+	Non-toxin gene bearing *C. ulcerans/ C. pseudotuberculosis* detected
-	+	+	+	Toxin gene bearing *C.ulcerans/ C. pseudotuberculosis* detected
-	-	-	+	*C. diphtheriae/ C. ulcerans/ C. pseudotuberculosis* not detected
-	-	-	-	*Inhibitory* PCR
-	-	+	+	Example of equivocal PCR needs to repeat
+	-	-	-	Example of equivocal PCR needs to repeat

Appendix 9: Chapter 6- Standard operation protocol: modified Elek test

Method:

1. Melt 2.5ml Elek agar medium and transfer to 50^0C water bath.
2. Add 0.5 ml sterile newborn bovine serum to the melted basal medium.
3. Mix carefully and pour immediately into a 5cm plate. Allow to set and then dry at 37^0C for 30 minutes. Do not over-dry.
4. Label the dried plate as per the template. Two test strains can be accommodated on one plate.
5. In a Class II safety cabinet and wearing gloves with a 1 ul loop, inoculate the plate with the two test strains and the three control strains as indicated on the template.
6. Using pre-flamed forceps, place a diphtheria antitoxin disc (10 IU/ml) on the plate as per template.
7. Remove the plate from the cabinet and swab the base of the cabinet with 70% alcohol. Discard the paper towels for incineration. If you have used the paper template in the cabinet, also discard this into the appropriate container. Remove your gloves and discard them in a container.
8. Incubate the plate in the 37^0C hot room for 16-24 hours only.

Reading Modified Elek Test (24-hour reading only)

1. Using a suitable light source and wearing gloves, examine the plate carefully after overnight incubation looking for precipitin lines of identity between the test strains and the strong and weak positive control strains. The negative control strain should not demonstrate any precipitin lines.
2. Reincubate the conventional plate only for a further 24 hours and read again as above. Do not reincubate for longer than 48 hours, as non-specific precipitin lines may develop. The modified plate should not be reincubated.

Appendix 10: Chapter 6 - Standard operation protocol: Serum elution from Dried blood spot

Blood sample collection method:

Blood will be collected using a 23G needle and 5 ml syringe by standard venipuncture. Blood will be spot on the filter paper (Whatman#903). The blood will be dried for at least 3 hours, avoiding direct sunlight or heat, then will be packed with silica gel and stored at room temperature or in a refrigerator, if available, until transported to the Pasteur Institute in Nha Trang.

When the Dried Blood Spot (DBS) is received at the laboratory, DBS will be stored in a -80 degree freezer immediately. Take out the sample in certain batches, such as 40 samples (samples collected in one village), punch out the DBS with 6mm punches, and store them in a -80 degree freezer until testing.

Serum elution from Dried blood spot (DBS):

Materials:

- Filter paper (Whatman #903)
- PBS Buffer (Sigma), Skim milk powder (Sigma-Aldrich), Tween 20 (100%) (Sigma-Aldrich)
- Filter tip 20ul, Unfilter tip 1000ul
- Eppendorf tube 1.5ml (elution of DBS), 5ml blood collection tube (dilution for ELISA)
- Diphtheria IgG ELISA kit (IBL), Tetanus IgG ELISA kit (IBL), Pertussis ELISA kit (Abcam)

Method:

1. Preparation for the filter paper: following the WHO DBS guide
2. Sample size for comparison of paired serum and a dried sample was based on Altman –Blant method and justified by the number used in the previous report.
3. On the day of the survey, serum samples and DBS will be collected. Samples will be stored in a -30 degree freezer until use.
4. DBS is punched out with a 6 mm hole punch and stored in Eppendorf tubes.
5. One day before the ELISA testing, elute DBS in a 1.5ml Eppendorf tube.
6. Elution method:
   6 mm diameter disc contains about 5ul serum

Create the elution solution[1] mixed with 250ml PBS +tween20 (0.05% solution) 0.125ml + skim milk 2.5g for eluting blood disc. (final concentration of tween20 is 0.05%, skim milk is 1%)

Soak the disc in the 500ul elution buffer to create 1:100 dilution.

Vortex 30 seconds. Incubate at room temperature for 15 minutes. Vortex 30 seconds again.

Incubate overnight under 4 ℃ refrigerator

7. The next morning, vortex the solution for 30 seconds and centrifuge for 15 minutes by 3800 rpm to spin down the broken paper. Use supernatant.
8. Process ELISA following the IBL (diphtheria and tetanus antitoxoid antibody) or Abcam (pertussis antitoxoid antibody) protocol. DILUTION is NOT NECESSARY before putting the samples on the plates.

Control: for the first run, use normal control and control diluted by elution solution[1] and compare the results (draw double standard curve).

9. Enter the results in excel in the dedicated folder. Check the results of control and all the data. If there are any unusual results, report them.
10. Data: draw a fitted cubic curve in excel. Solve cubic equation in R using the parameter obtained from the cubic curve.

Elution solution: stored in a refrigerator

PBS buffer	250ml	500ml
Tween20 (100%)	125ul (0.05%)	250ul (0.05%)
Skim milk (powder)	2.5g (1%)	5g (1%)

Appendix 11: Chapter 6- Standard operation protocol: Diphtheria anti-toxoid IgG detection by ELISA

Appendix 11-1. Anti-diphtheria toxoid IgG ELISA kit (IBL)

Material

- Micropipettes (Multipette Eppendorf or similar devices, < 3 % CV). Volumes: 5; 50; 100; 500 µL
- Calibrated measures
- Tubes (1 mL) for sample dilution
- 8-Channel Micropipettor with reagent reservoirs
- Wash bottle, automated or semi-automated microtiter plate washing system
- Bidistilled or deionized water
- Paper towels, pipette tips, and timer
- Diphtheria IgG ELISA Kit, IBL.

Pre-test setup instruction

Reagent preparation

- Take out all reagents, samples, and controls to room temperature (18-25°C) and gently swirl each vial of liquid reagent and sample before use.

Preparation of Components:

- 20 ml of WASHBUF CONC was diluted with 180 ml of bidistilled water (ratio 1:10). Warm up at 37 °C to dissolve crystals, if necessary. Mix vigorously. Storage at 2-8ºC for 8 weeks (maximum).

Dilution of samples:

- Dilute samples with the ratio of 1:101 (e.g., 5µl of sample + 500 µl of DILBUF)

Methods:

1. Pipette 100 µL of each Standard and diluted serum or plasma sample into the respective wells.
2. Cover plate with adhesive foil. Incubate for 60 min at 18-25 °C.
3. Remove adhesive foil. Discard incubation solution. Wash plate 3 x with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
4. Pipette 100 µL of Enzyme Conjugate into each well
5. Cover plate with new adhesive foil. Incubate for 30 min at 18-25 °C.

6. Remove adhesive foil. Discard incubation solution. Wash plate 3 x with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
7. For adding substrate and stop solution, use, if available, an 8-channel micropipettor. Pipetting. should be carried out in the same time intervals for Substrate and Stop Solution. Use positive displacement and avoid the formation of air bubbles.
8. Pipette 100 µL of TMB Substrate Solution into each well.
9. Incubate for 20 min at 18-25 °C in the dark (without adhesive foil).
10. Stop the substrate reaction by adding 100 µL of TMB Stop Solution into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow
11. Measure optical density with a photometer at 450 nm (Reference-wavelength: 600-650 nm) within 60 min after pipetting of the Stop Solution.

Calculation of Results

- Plot the obtained OD of the standard samples (y-axis, linear) against their concentration (x-axis, logarithmic or linear) and draw a standard curve by point-to-point.
- Calculate the concentration of each sample from the standard curve.
- Results of samples of higher predilution have to be multiplied with the dilution factor.
- Samples showing concentrations above the highest standard have to be diluted as described in the Pre-test setup instructions and reassayed.

In order for an assay to be considered valid, the following criteria must be met:

	Concentration IU/ml	Acceptable range OD
CAL A	0.0	0.001 - 0.150
CAL B	0.01	0.030 - 0.200
CAL C	0.1	0.150 - 0.750
CAL D	0.5	0.400 - 2.500
CAL E	1.0	0.750 - 3.000

Appendix 11-2. VaccZyme Diphtheria Toxoid IgG Enzyme immunoassay kit: MK014 (Binding site)

Pre-assay steps:

1. Bring the kit to room temperature. Do not open the foil bag of the wells (plates). Keep wells for about 1 hour at room temperature.
2. Gently mix kit components before use.
3. Add 50ml wash buffer to 950ml distilled water (1:20 dilution). Diluted buffer can be stored at room temperature for 4 weeks.
4. Do not need to dilute samples when DBS is eluted, as they are already diluted by 1:100.

Assay method:

1. Add 100ul of 7 calibrators (toxoid antibody: 3, 1, 0.333, 0.111, 0.037, 0.012, 0.004 IU/ml), and high and low control and each sample (eluted DBS) into the appropriate well. Samples should be added as quickly as possible so that incubation time will be equal for all samples.
2. Incubate at room temperature for 30 minutes.
3. Wash wells 3 times with 250-350ul wash buffer per well.
4. After the final wash, invert the plate and tap the wells dry on absorbent paper.
5. Add 100ul of conjugate into each well. Blot the top of the wells with tissue to remove any splashes. (using 8-channel pipet)
6. Incubate at room temperature for 30 min.
7. Wash wells 3 times with 250-350ul wash buffer per well.
8. After the final wash, invert the plate and tap the wells dry on absorbent paper.
9. Add 100ul of TMB substrate into each well. Blot the top of the wells with tissue to remove any splashes. (using 8-channel pipet)
10. Incubate at room temperature in the dark (put the foil on top of the plates) for 30 min.
11. Add 100ul of stop solution into each well. This causes a change in colour from blue to yellow.
12. Read the optical density (OD) of each well at 450 nm on a microplate reader within 30 minutes of stopping the reaction.

Calculate concentration:

1. Type ID and OD on excel. Calculate the concentration (IU/ml) based on the standard curve fitted by four parametric logistics.

2. Check the value of high control within 0.58-0.96 IU/ml and low control within 0.11-0.23 IU/ml. If the value is out of range, report. (after consultation, the run will need to be repeated)
3. Store the excel sheet in a designated folder.

Plate map:

Con 3	Con Low										
Con 1	Sample 1										
Con 0.333	Sample 2										
Con 0.111	Sample 3										
Con 0.037	..										
Con 0.012	..										
Con 0.004	..										
Con High	..										

Appendix 12: Chapter 6- Exposure variables

a)	Age, sex, and residence village	
b)	Recent respiratory symptoms, skin lesions and antibiotics use	
c)	Past medical history:	chronic tonsillitis, goitre, heart disease
d)	Ethnicity:	Co, Hre, Xo Dang, and Kinh (253)
e)	Vaccination history	
f)	Human to human contact	
	Household size, Number of children in the household	
	Living conditions:	sharing bed with 2 or more persons
		sharing bed/room with 2 or more persons
	Attendance at school: nursery, primary school, secondary school, high school, college	
	Use of school dormitory: number of persons in one dormitory	
	Contact with a person with skin lesions at home or in dormitory	
g)	Animal to human contact:	
	Number of domestic animals	pigs, cattle, horses, goats, sheep, dogs and cats
h)	Mid-upper arm circumstances (MUAC) in pre-school age group	
i)	Smoking, hand washing and bathing	
j)	Socio-Economic Status:	
	a. Cooking method	wood, charcoal, gas
	b. Water source	tap, well, open-water
	c. Toilet facilities	

Ethnic groups in Quang Ngai province were identified from open source. (253)

Appendix 13: Chapter 6- Survey questionnaire

2	Survey ID:	e.g. 1-15-1001, put the label		
3	Sex:	1.Male	2.Female	
4	Date of Birth: (dd/mm/yy)			
4a	If the person does not remember the DOB, Age (year):			
5	Did participant receive diphtheria contained vaccine before? E.g. 5 in 1 or DPT	1.Yes	2.No	3.Unknown
	If yes in 5, which diphtheria vaccine did participant receive?			
5a	DTP	1.Yes	2.No	3.Unknown
5b	5 in 1 (e.g. children born after 2010)	1.Yes	2.No	3.Unknown
5c	6 in 1 (e.g. private clinic may provide this)	1.Yes	2.No	3.Unknown
6	Did participant show the vaccination card to CHC staff to answer below?		1.Yes	2.No

Routine Vaccination History Please write the vaccinated date. (e.g. 15/04/2019, dd/mm/yyyy)
If date is unknown, fill the cell with "Yes (Y)" vaccinated, "No (N)" not vaccinated, or "Unknown (UK)".

		Y/N/UK	Birth-dose	1st (Date)	2nd (Date)	3rd (Date)	4th (Date)
7	BCG						
8	HepB (or 5/6in1)						
9	DTP (or 5/6in1)						
10	Hib (or 5/6in1)						
11	OPV						
12	Measles (or MR)						
13	Rubella (or MR)						
14	Japanese Encephalitis:						
15	Was the participant registered in this commune (Name of commune:)?					1.Yes	2.No
16	If Q15 is **YES**, **AND** children is younger than <=10 years old, **AND** did not bring the vaccine card, Did CHC staff find the participant's record on their registration book? If yes, fill the date above Q7-14 copying from the registration book.					1.Yes	2.No
15b	Was the participant born as a premature baby (before 37 gestational weeks)?					1.Yes 2.No	3.Unknwon

If other childhood vaccines outside of EPI program (e.g. TdaP, PCV, Rota, Influenza) were given, please specify the name and Date of given.

	Name of vaccine	1st (Date)	2nd (Date)	3rd (Date)	4th (Date)
17					
18					
19					

19a						

Did participant receive below additional vaccine? (If age was unknown, fill 99 in age. Alternatively, put X years ago)

20	MR campaign	1.Yes	2.No	3. Unknown	20a If yes, at what age?	
21	OPV campaign	1.Yes	2.No	3. Unknown	21a If yes, at what age?	
22	dT campaign	1.Yes	2.No	3. Unknown	22a If yes, at what age?	
23	TT during pregnancy	1.Yes	2.No	3. Unknown	23a If yes, at what age?	
					[illegible]	
24	TT at injury/trauma	1.Yes	2.No	3. Unknown	[illegible]	

Past Medical History

Diphtheria

25	Has participant ever been diagnosed with **diphtheria** before?	1.Yes	2.No	3.Unknown
25a	If No, skip to 26.1. If yes in 25, at what age?		years (if unknown, put99)	
25b	How was participant diagnosed? 1.Lab diagnosis		2.Clinical diagnosis	3. Other or unknown
25c	Where was participant diagnosed?(name of province)			
25d	Has participant diagnosed more than once?	1.Yes	2.No	
25e	If yes in 25d, how many times?			

Pertussis

26.1	Has participant ever been diagnosed with **pertussis** before?	1.Yes	2.No	3.Unknown
26.2	Has participant had persistent cough and had one of following symptom, Paroxysms of coughing, OR Inspiratory "whoop," OR Apnea (if infant) before?	1.Yes	2.No	3.Unknown
26a	If No in 26.1 or .2, skip to 27.1. If yes, at what age?		years (if unknown, put99)	
26b	How was participant diagnosed? 1.Lab diagnosis		2.Clinical diagnosis	3. Other or unknown

Tetanus

27.1	Has participant ever been diagnosed with **tetanus** before?	1.Yes	2.No	3.Unknwon
27.2	Has participant had "lock jaw" or "spasm" of the muscle and/or neck stiffness during 3-28 days old or at the time of injured?	1.Yes	2.No	3.Unknwon
27a	If No in 27.1 or .2, skip to 28. If yes, at what age?		years (if unknown, put 99)	
27b	How was participant diagnosed?		2.Clinical diagnosis	3. Other or unknown

Family History of Diphtheria, Pertussis, and Tetanus

28	Did your family has either Diphtheria, Pertussis or Tetanus before?	1.Yes	2.No	3.Unknown

If 28 is "No" or "Unknown", skip to 34.

	a. Disease	b.Who is that?	c. Age of that person	d. Age of diagnosis	e. Diagnostic Method	f. Where was s/he diagnosed (name of

	(Diphtheria, Pertussis, or Tetanus)	(Relationship with participant)		(year/month)		province or country if outside of Vietnam)
29					1.lab/2.clinical/3.others	
30					1.lab/2.clinical/3.others	
31					1.lab/2.clinical/3.others	
32					1.lab/2.clinical/3.others	

Travel history								
34	Have you traveled or lived to/in below Provinces since 2012?				1.Yes		2.No	
a	Quang Nam	c	Kon Tum		e	Binh Phuoc		
b	~~Quang Ngai~~	d	Gia Lai		f	HCMC-Can Gia District		
35	Have you traveled or lived to/in below countries since 2012?				1.Yes		2.No	
a	Indonesia	d	India		g	Myanmar		
b	Philippine	e	Cambodia		h	Bangladesh		
c	Thailand	f	Lao PDR		i	Malaysia		
36a	Has participant met and talked more than 5 minutes with any person who came from the areas listed in 34 and 35 since 2012?				1.Yes	2.No	3.Unknown	
36	Has participant got contact with diphtheria suspected or confirmed case before?				1.Yes	2.No	3.Unknown	
42	If participant is older than 5 years old, skip to 43. If participant is 1-5 years of age, is participant attending nursery?				1.Yes □ go to 47		2.No □ go to 48	
43	Is participant attending school? If NO, go to 48.				1.Yes		2.No	
44	What grade is participant in? (Answer 1 to 12)							
45	Does participant's school have dormitory?				1.Yes		2.No	
46	Is participant staying at the dormitory or house for nap with other students?				1.Yes		2.No	
47	If yes in 46, how many students are staying in your dormitory room?							
	1.<10 pps	2.10-19 pps	3.20-29 pps	4.30-39 pps	5.40-49 pps	6.50-59 pps	7.>=60 pps	
48	How many people are staying in the participant's house including participant (regularly)? (number)							
49	Is there any household member who is younger than 18 years of age?				1.Yes		2.No	
50	If yes, how old is he/she and does he or she go to school or nursery? List all member's age and school attendance.							
	Child	1	2	3	4	5	6	
	Age (year)							
	School or nursery Attended?	1.Yes 2.No	1.Yes 2.No	1.Yes 2.No	1.Yes 2.No	1.Yes 2.No	1.Yes 2.No	
51	Is participant sharing a bed with more than one person when s/he sleeps (>=2 persons is sleeping in 1 bed)?				1.Yes		2.No	
52	If yes, who are you sharing the bed? (select all applicable choice)		1. Child <=5 yrs	2. Child 6-17 yrs	3. Adults 18-40 yrs		4. Adults >40 yrs	

53	Is participant sharing a room with more than one person at the house when s/he sleeps (>=2 persons is sleeping in 1 room)?					1.Yes	2.No
54	If yes, how many people are sharing one room? (number)						
54a	If yes, who are you sharing the room? (select all applicable choice)	1. Child <=5 yrs	2. Child 6-17 yrs			3. Adults 18-40 yrs	4. Adults >40 yrs
54b	Did you share the utensils at home (chopsticks, spoons, dishes)?					1.Yes	2.No
55	What is your ethnicity?	1. Co	2. Hre	3. Xo Dang	4. Kinh	5.Other(specify): _____	
56	Is participant a farmer, or engaging farming for living? Skip for less than 12 yo.					1.Yes	2.No
57	Did or do participant have a skin disease which is similar to the photo in last one month? (Please show the photo to the participants)					1.Yes	2.No
57a	Did participant's family or roommate in school dormitories have skin lesions in last one month? (Please show the same photo to the participants)					1.Yes	2.No
58	Did participant have any respiratory symptom (cough, sore throat, etc) in last one month?					1.Yes	2.No
58a	Did participant have fever "and" muscle pain in last one month?					1.Yes	2.No
58b	Did participant have any chronic illness?	1.chronic tonsillitis				2. goiter	3. heart problem
	4. other. Please specify _____	5. Nothing					
59	Did or has participant been taking any antibiotics (such as, erythromycin, amoxicillin, or penicillin) in last one month?					1.Yes	2.No
60	Do you smoke?	1.Regularly (>1/week)	2. Sometimes (< =1 / week)			3. In the past	4. Never
60a	If yes (participant answer 1,2, or 3 in 63), how long did participant smoke? If less than a year, put 0.5.						year
61	Does any of your household member smoke?					1.Yes	2.No
62	Did participant wash his/her hand with soap and water yesterday?	1.Yes, with soap and water				2.Yes, but water only	3. No
62a	If yes, how many times did participant wash hand yesterday?						times
63	Did you bathe or shower last one week?					1.Yes	2.No
63a	How many times did you bathe or shower last one week (number 1 to 7)						times

HH Questionnaire: Only one person (e.g., first member) in each household should answer below

64	What is the main composition of the walls of the dwelling? (2015 census: SES 3)			
	1. Cement	2. Bricks	3. Wooden Planks	4. Mud bricks,
	5. Tin	6. Sticks	7. Other (specify) _____	
65	How large is your house (in square meters)? _____ m2 . If the participant has no estimation, put (1) (SES4)			
66	What is the water source in your house (multiple choices are allowed)? (SES6)			

	1.Tap	2.Water Truck/Boat	3.Tube well / hand pump	4. Open well
	5. Rain Water	6. Canal/River	7. Lake/Pond	8.Other(specify) _____
67	Does your family have toilet? (SES 9)		1.Yes	2.No
68	If yes, what kind of toilet do members of your household usually use?		1.pit toilet	2.flush toilet
69	Is your kitchen inside your house? (SES10)		1.Yes	2.No
70	What is your main source of energy for cooking? (SES11)			
	1.Gas	2.Coal	3.Oil	4.Bio fuel (Animal's faces, Wood, straw)	5.Other (specify)
71	In your household compound, How many following animals or pets are there? (SES14) Put the number of animas in the bracket [____]. If there is no animals, please put "0". If there are too many and cannot count, put "99".			
	1. Pigs [____]	2.Buffalos [____]	3. Dogs [____]	4. Cows [____]
	5. Cats [____]	6. Chickens or birds [____]	7. Ducks/Geese [____]	8. Others: _____ [____]

The below information was not entered in the database except 38.

37	Date of interview:	__/ __/2019			
38	Name of participant:				
39	Interviewer's name:			Signature:	
41	Mid Upper Arm Circumferences (MUAC) for children 1-5 years old				mm
Sample collected:					
Throat swab		Nasopharyngeal swab		Dried Blood Spot	
Yes	No	Yes	No	Yes	No

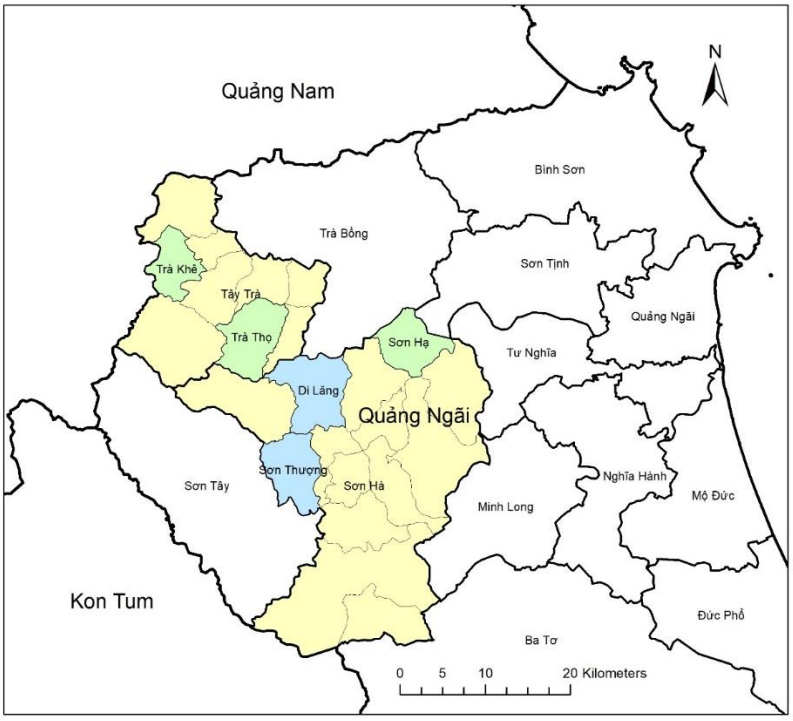
A written questionnaire was developed, translated from English to Vietnamese, and translated back to English by a second person to verify the translation. The questionnaire was piloted by two surveyors with five participants before the survey.

Appendix 14: Chapter 6-Survey method

Appendix 14-1. Sampling method

Basic information of administrative units in Tay Tra and Son Ha district in Quang Ngai province

	Tay Tra	Son Ha
SIA	October 2019	October 2019
Population	20,379	74,463
Number of communes	9	14
Total number of villages (sub-section in commune)	36	101
Average population in 1 commune	2,264	5,319
Average population in 1 village	566	737



Multistage sampling

We will conduct multi-stage cluster sampling in Tay Tra and Son Ha districts in Quang Ngai. The primary sampling unit will be the commune, and the cluster unit will be the village. The final sampling unit will be the household. From each district, we will select five communes by population proportion to size sampling (PPS) and will randomly select based on a simple random sampling of three villages from each commune. First, we will obtain the population list for the communes. PPS will be started in one commune where the cumulative population is matched to a generated random number. The next commune will be selected by the serial interval (each commune population divided by five) to obtain a total of five communes.

Random selection of the first village will be made by the random number generator function in STATA. The villages allocated the three smallest numbers by this function will be selected from 1 to n (where n is the number of villages in the commune). A total cluster of 30 will be selected across the two districts. The selected communes and villages are listed in the table below.

Table 2.

No	District	Commune	village/hamlets		
1	Tay Tra	Tra Phong	Tra Bung	Na	Tra Nga
2		Tra Quan	Tra Ong	Tra Bao	Tra Xuong
3		Tra Thanh	Thôn Gỗ	Thôn Môn	Thôn Vuông
4		Tra Lanh	Tra Luong	Tra Ich	Tra Dinh
5		Tra Xinh	Tra Oi	Tra Kem	Tra Veo
1	Son Ha	Son Ha	Đeo Ron	Đong Reng	Ha Bac
2		Son Nham	Canh Mo	Cham Rao	Xa Rieng
3		Son Giang	Go Ngoai	Lang Ri	Ta Dinh
4		Son Ky	Re	Rut	Nuoc Lac
5		Son Hai	Lanh	Ren	Trang

The average household size in Vietnam is four (MICS 2014). If we plan to recruit 1500 subjects between the age of 1 and 55 years in Quang Ngai Province, we need to recruit 450 households estimated on the basis of the population of Nha Trang city (85% of the total population is between 1 and 55 years old).

Table 3. shows the number of recruitment and the final calculated sample size required by age in total and per cluster.

Table 3.

	Recruitment	Recruitment per cluster	final sample size (80% response)	final sample size per cluster (80% response)	Percentage of each age group among total population
Total sample	1500	50	1200	40	100%
1-5 yr	350	12	280	9	23%
6-17 yr	400	13	320	11	27%
18-40 yr	400	13	320	11	27%
41-55 yr	350	12	280	9	23%
number of households	450	15	300	10	

The target population by age group in the two districts is summarised in Table 4.

Table 4.

Age group	Tay Tra	%	Son Ha	%	Two districts	%
<=5 yr	2,154	11%	7,494	10%	9,648	10%
6-17 yr	7,110	35%	15,787	21%	22,897	24%
18-40 yr	11,015	54%	24,278	33%	35,293	37%
>=41 yr	5,150	25%	74,463	36%	32,054	34%
Total	20,379	100%	26,904	100%	94,842	100%

We assume random household sampling will recruit the subject proportionally to the required numbers for three age groups above five years of age. However, if the total number of people aged over 18 years (18-40 and 41-55 yr) exceeds 20 per cluster, we stop recruiting these age groups and continue recruiting children. We will stop recruiting if 6-17 years of participants reach 13 per cluster and will recruit 1-5 years. For the 1-5 years age group, we conduct oversampling. Five households per cluster will be randomly selected from households with children 1-5 years old, and 10 remaining households will be randomly selected from all households.

Appendix 14-2. Bias

We did not conduct a household survey; therefore, the selection of the participants may be biased. For example, participants living close to the CHC will be more likely to join the study and may be more likely to receive vaccination as a routine health service. To reduce this selection bias, the research staff will select a community meeting room or equivalent venue, which is located near the centre of the selected village and convenient for most residents to visit, as the survey site.

Appendix 14-3. Sample size calculation

Sample size is calculated for measuring age-stratified seroprevalence based on the following formula:

$$n = \frac{\mathrm{Z}^2 \mathrm{p}(1-\mathrm{p})\mathrm{DEFF}}{\mathrm{RR}\, d^2}$$

where

n = sample size for single stratum

Z= significance level for 95% confidence interval (1.96)

p= expected prevalence (70, 40, 40, 30%)

d= precision (10%)

DEFF= design effect (3.5)

RR= response rate (80%)

Population is divided into four age strata: 1-5, 6-17, 18-40, and 41-55 years of age. The previous survey conducted in 2015 in Kontum province (neighbouring province) indicated the seroprevalence of diphtheria among people aged 6 to 25 years was 40%. Another survey in Khanh Hoa province showed seroprevalence of 68% at 1-5, 6% at 6-17, 23% at 18-40, and 32% at 41-55 years of age. Therefore, prevalence estimates of 70%, 40%, 40%, and 30% were used for sample size estimation by age group, 1-5, 6-17, 18-40, and 41-55 years, respectively.

Sample size is determined for 1-5 years: 350, for 6-17 years: 400, for 18-40 years: 400, for 40-55 years: 350 (Total 1500), accounting for the 80% response rate.

Appendix 15: Chapter 7- Standard Operation Protocol: Vero Cell Asay: the determination of diphtheria antitoxin in serum samples (UK-HSA protocol)

Principle:

The diphtheria antitoxin Vero cell toxin neutralization assay is based on the capacity of diphtheria toxin to block protein synthesis and cause cell death in cultured mammalian cells and the neutralization of this effect when diphtheria antitoxin antibodies re present in serum specimens. The first dilution at which toxin neutralization is reported is the antibody level in international units per milliliter (IU/ml).

Materials:

- Minimum Essential Medium (MEM)
- Diphtheria toxin

Add 50µl of 50Lf/ml diphtheria toxin stock solution to 4.95ml prepared MEM and make further dilution as 75µl of them in 20ml of MEM.

- Diphtheria antitoxin (3rd British Standard)

Obtained from the National Institute for Biological Standards and Control (NIBSC), UK.

50µl of 5.0IU/ml diphtheria antitoxin stock solution is added to 2.45 ml prepared MEM. Then, 2.0ml of the above 0.1IU/ml dilution is added to 1.12 ml prepared MEM producing a 0.064IU/ml solution for use in the Vero cell assay.

- Vero cell suspension in MEM containing 2.5 x 10^5 cells/ml

Vero (ECACC catalogue no. 84113001) cell line is a continuous and aneuploid cell line of mammalian origin. Vero is susceptible to infection from a number of viruses and bacterial toxins such as diphtheria toxin or Shiga-like toxin. Vero cells must be maintained at least once a week. For the assay, the Vero cells must be between 3 and 7 days old.

- Human sera containing a known concentration of antitoxin antibodies

Archived human sera that have previously been tested in the assay are used as internal quality control samples. Ideally, samples should have antibody levels of <0.008, 0.016, 0.128, and >1.0IU/ml to test the specificity and reproducibility of the test.

- Serum samples

The quality of the sample can have an effect on the result. Samples containing red blood cells can make the test difficult to read as they settle over the Vero cell monolayer.

Lyzed samples or samples with bacterial contamination can be toxic to the Vero cells

Samples with high levels of toxin neutralising antibodies can be toxic to the Vero cells, and a prozone effect will be seen.

The patients should not have undergone any treatments, such as dialysis, which removes any antibodies.

- Calibrated 8-channel pipette pre-set to 50µl and appropriate sterile tips
- Sterile 96 well Greiner tissue culture plates
- BIS sealing pressure film (the glue used on the other sealer may become opaque during incubation making the test impossible to read)
- Reagent reservoirs
- Calibrated pipetts, 50 µl, 75µl, 100µl, and 1000µl with sterile tips
- Sterile plastic bijoux
- Sterile plastic universal bottles
- Sterile single-wrapped 25ml and 10ml pipettes
- Pipette aid

Methods:

- Gloves and safety glasses must be worn when handling serum samples and media.
- Serum samples are stored between 2 to 8 ℃.
- Diphtheria toxin and antitoxin must be freshly prepared before the assay is performed.
- Worksheet and template are completed with the sample and reagent batch information.
- Add 50µl antitoxin (0.064IU/ml) to wells G1 and H1.
- Add 50µl test serum to wells A1, B1, C1, D1, and E1 and again to wells A11, B11, C11, D11, and E11, according to the worksheet.
- Add 50µl control serum to wells F1 and F11.
- Add 100µl prepared MEM to wells G10 ad H10.
- Pipette 50µl prepared MEM in all wells of the 96-well microtitre plate (starting at column 12) using an 8-channel pipette.
- Using the 8-channel pipette and beginning at column 1, perform doubling dilutions of 50 µl volumes across the microtitre plate up to and including column 7.
- Remove tips for rows G and H after column 7.
- Continue doubling dilutions in rows A to F up to and including column 10.
- Discard 50µl from column 10 (rows A-F).
- Using the same tips, continue diluting from column 11 to column 12.
- Discard 50 ul from column 12 after mixing.
- Add 50µl of toxin to all wells up to and including column 9, and then remove tips, add toxin to rows G and H, and continue adding toxin to column 10, rows A to F.

- Do not add toxin to columns 11 and 12
- Add 50µl prepared MEM to wells 11A-H and 12 A-H.
- Replace plate lids and incubate for at least 1 hour at 35-37℃.
- Using 2 ml of suspended Vero cells, make 1 in 11 dilutions (2ml of cells plus 20ml of prepared MEM) to give a cell suspension of approximately 2.5 x 10^5 cells/ml.
- Add 50µl of cell suspension to all wells except G10 and H10.
- Seal plates with BIS plate sealers (sticky lids) and incubate for 3-4 days at 35-37 ℃. Plates can be read up to a maximum of 7 days incubation.
- Assay plates are read microscopically to check for cell growth, the absence of contamination, and for serum toxigenicity.
- Turn the light to full and focus on the cell growth in the plate
- Results are recorded on the worksheet.

Quality control:

- A graphical record is kept of the results for each internal quality control samples
- Results must not differ by more than one doubling dilution for a valid test
- If the result of an individual control serum fails to meet the criteria, the entire plate must be repeated with a new control sample aliquot.
- If the results of all four control sera differ by more than one doubling dilution, the entire test must be repeated.
- To maintain the sensitivity of the test, cell growth should be seen in the first 4 wells of the reference antitoxin. If growth is seen in the first 5 wells, the in-use toxin is too weak and the volume should be increased in the toxin preparation step. If growth is seen in the first 3 wells only, the in-use toxin is too strong, and the volume should be reduced in the toxin preparation step.
- Controls for cell growth, toxin activity and serum cytotoxic activity are incorporated into each plate.
- Ensure that the control sera have given the correct result and these results are recorded on the worksheet, including that for the internal positive controls data trending graph.

Interpretation of the results:

- Antitoxin antibody concentrations of the samples are calculated in IU/ml by taking the last dilution of serum at which cells grew and multiplying the dilution factor by the lowest.
- Current internationally accepted criteria for interpretation of diphtheria serum antitoxin levels are as follows:

Antitoxin level	Interpretation
<0.01 IU/ml	individual is susceptible
0.01 IU/ml	lowest leve of circulating antitoxin giving some degree of protection
0.01-0.09 IU/ml	levels of antitoxin giving some degree of protection
0.1 IU/ml	a protective level of circulating antitoxin
≥ 0.1IU/ml	a level of antitoxin giving Long-term protection

Neutralization dilution	Dilution factor	Toxin antibody titre IU/ml	International interpretation
1	0.004	0.004	≤0.008 IU/ml Indicating susceptibility
2	0.004	0.008	
4	0.004	0.016	0.016-0.09 IU/ml Basic protection
8	0.004	0.032	
16	0.004	0.064	
32	0.004	0.128	≥0.128 IU/ml Full immunity
64	0.004	0.256	
128	0.004	0.512	
256	0.004	1.024	≥1.0 IU/ml Long-term protection
512	0.004	2.048	

Plate layout for diphtheria neutralization assay

	Serum Dilution										No toxin	
	2	4	8	16	32	64	128	256	512	1024	2	4
A	S T	S T	S T	S T	S T	S T	S T	S T	S T	S T	S	S
B	S T	S T	S T	S T	S T	S T	S T	S T	S T	S T	S	S
C	S T	S T	S T	S T	S T	S T	S T	S T	S T	S T	S	S
D	S T	S T	S T	S T	S T	S T	S T	S T	S T	S T	S	S
E	S T	S T	S T	S T	S T	S T	S T	S T	S T	S T	S	S
F	IQC T	IQC T	IQC T	IQC T	IQC T	IQC T	IQC T	IQC T	IQC T	IQC T	IQC	IQC
G	aT T	aT T	aT T	aT T	aT T	aT T	aT T	Toxin only	Toxin only	MEM only	Cells only	Cells only
H	aT T	aT T	aT T	aT T	aT T	aT T	aT T	Toxin only	Toxin only	MEM only	Cells only	Cells only
	0.032	0.016	0.008	0.004	0.002	0.001	0.0005	Toxin Control		Blank	Cell Control	
	Antitoxin concentration IU/ml											

S: Test sample, T: Toxin, aT: antitoxin, IQC: Internal Quality Control