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Epidemiological and serological investigations of vaccine-preventable diseases and their implications for vaccination policy in the Lao People's Democratic Republic

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A tray full of money is not worth a mind full of knowledge

Lao proverb

TABLE OF CONTENTS

ABSTRACT

The Lao People's Democratic Republic (PDR) is a landlocked country in South-East Asia. Founded in 1975 by the Lao People's Revolutionary Party, the Lao PDR of today is a relatively young nation. The Expanded Program on Immunization of the World Health Organization was established in the country in 1979. Starting with a limited vaccination schedule including six vaccines (Bacillus Calmette-Guerin, Diphtheria, Tetanus, Pertussis, Polio, and Measles vaccine), the programme was extended during the following years and now also includes vaccines against hepatitis B virus, rubella virus, pneumococcal disease, Japanese encephalitis virus and human papilloma virus. The Expanded Program on Immunization is generally considered as one of the most successful health programs in the Lao PDR but still faces many challenges.

This research thesis describes my serological and epidemiological investigations of vaccine-preventable diseases and vaccination efforts in the Lao PDR with the aim to inform vaccination policy. Specifically, the overall objectives of this research work are to (i) characterize the epidemiology of vaccine-preventable diseases for which there is no routine vaccination, to (ii) assess the level of protection against vaccine-preventable diseases and investigate possible risk factors for seroprotection and (iii) evaluate the impact of vaccination on disease prevalence. The overall objectives were broken down into smaller research questions addressed in six research articles and one review article presented in the thesis. Three of the presented articles were already published in peer-reviewed journals.

In the first two articles, serological testing was used to establish baseline data for the prevalence of *Haemophilus influenzae* type b and *Salmonella enterica*, subspecies enterica serovar Typhi in the Lao PDR in absence of routine vaccination. The data may serve as reference for follow-up studies and/or as complementary data to clinical data.

Serological data was used to assess the level of population immunity in children after routine childhood vaccination with the pentavalent Diphtheria-Tetanus-Pertussis-Hepatitis B-*H. influenzae* type b vaccine and the measles and rubella vaccine. The studies highlighted the progress made but also identified predictors associated with suboptimal vaccination rates in Bolikhamxay, a central province in the Lao PDR. Furthermore, the timeliness of the vaccination was assessed and factors associated with delayed vaccination were identified.

The last two research articles dealt with the epidemiology of hepatitis B in the Lao PDR. A literature review was conducted to give an overview over the current data situation of hepatitis B prevalence in the country. In addition, a study with the aim to investigate the serological hepatitis B profiles among adolescents born before and after the introduction of the hepatitis B vaccination was conducted.

In summary, the data presented in this thesis contribute to the understanding of vaccine-preventable diseases in the Lao PDR, provide information regarding the vaccination efforts in the country and the

progress which has been made in the past years. The findings of these studies were translated into vaccine policy recommendations which were communicated to local public health authorities.

ZUSAMMENFASSUNG

Die Demokratische Volksrepublik Laos ist ein landumschlossener Binnenstaat in Süd-Ost Asien, welcher in 1975 durch die Laotische Revolutionäre Volkspartei gegründet wurde. Das erweiterte Programm für Immunisierung der Weltgesundheitsorganisation wurde in 1979 in der Demokratische Volksrepublik Laos etabliert. Zu Beginn des Programms verfügte der Impfkalender über sechs Impfstoffe gegen Tuberkulose, Diphtherie, Tetanus, Keuchhusten, Kinderlähmung und Masern. In den darauffolgenden Jahren wurden schrittweise weitere Impfungen hinzugefügt. Zum aktuellen Impfkalender gehören demnach Impfstoffe gegen Hepatitis B, *Haemophilus influenzae* Typ b Infektionen, Pneumokokken, Röteln, Japanische Enzephalitis und gegen das Humane Papillomavirus. Das erweitere Programm für Immunisierung wird als eines der erfolgreichsten Gesundheitsprogramme in der Demokratischen Volksrepublik Laos angesehen, ist allerdings immer noch mit großen Herausforderungen konfrontiert.

Diese wissenschaftliche Arbeit beschreibt meine serologischen und epidemiologischen Untersuchungen der Prävalenz von durch Impfungen vermeidbare Infektionskrankheiten und von der aktuellen Lage der Impfbemühungen in der Demokratischen Volksrepublik Laos mit dem Ziel, Daten zu erheben, mit deren Hilfe Impfstrategien entwickelt und verbessert werden können.

Die übergeordneten Ziele dieser wissenschaftlichen Arbeit waren (i) die Epidemiologie von durch Impfungen vermeidbaren Krankheiten zu charakterisieren bevor die Impfung im Land eingeführt wurde, oder für die es noch keine Impfung im Land gibt, (ii) das Ausmaß des Impfschutzes zu bewerten und mögliche Risikofaktoren für Seroprotektion zu untersuchen und (iii) die Auswirkung von Impfungen auf die Prävalenz von Infektionkrankheiten zu beurteilen. Diese drei Ziele wurden in spezifischere Forschungsfragen aufgegliedert, welche in einzelnen, zielgerichteten Studien adressiert wurden und welche auf die Bedürfnisse des Landes zugeschnitten sind. Die Ergebnisse der durchgeführten Studien sind in der Form von sechs Forschungsartikeln und einem Übersichtsartikel in dieser Doktorarbeit präsentiert. Drei der hier vorgestellten Studien wurden bereits in referierten Fachzeitschriften veröffentlicht.

In den ersten zwei Forschungsartikel wurden mit Hilfe von serologischen Tests eine Ausgangsbasis für die Prävalenz in der allgemeinen Bevölkerung von Antikörpern gegen *Haemophilus influenzae* Typ b vor der Einführung der Impfung und von Antikörpern gegen *Salmonella enterica*, subspecies enterica serovar Typhi, gegen welches es noch keine Impfung in Laos gibt, geschaffen. Die erhobenen Daten können als Referenz für nachfolgende Studien und/oder als Ergänzung zu klinischen Daten dienen.

Serologische Daten wurden zudem genutzt, um das Ausmaß des Impfschutzes in Kindern nach der Routineimpfung mit dem fünffachen Impfstoff gegen Diphtherie, Tetanus, Keuchhusten, Hepatitis B und *Haemophilus influenzae* Infektion und mit dem Masern-Röteln Impfstoff zu untersuchen. Die

Studien heben die Fortschritte hervor, welche in den letzten Jahren hinsichtlich des Impfschutzes gemacht wurden, identifizierten allerdings auch Faktoren, welche mit suboptimalem Impfschutz in Bolikhamxay, einer zentralen Provinz in Laos, in Verbindung stehen. Des Weiteren wurde der Zeitpunkt der Impfung untersucht und Faktoren identifiziert, welche mit verspäteter Impfung im Zusammenhang stehen.

Die letzten zwei Artikel in dieser Arbeit untersuchen die Epidemiologie von Hepatitis B in der Demokratischen Volksrepublik Laos. Eine Literaturrecherche wurde durchgeführt, um eine Übersicht über die aktuelle Datenlage hinsichtlich Hepatitis B in Laos zu erstellen. Die Ergebnisse wurden in einem Übersichtsartikel dargestellt. Zusätzlich dazu wurde eine Feldstudie durchgeführt um die Prävalenz von Hepatitis B Infektionen in Jugendlichen, die kurz vor und in den Jahren nach der Einführung der Hepatitis B Impfung in Laos geboren wurden, zu untersuchen.

Die Daten, welche in dieser Doktorarbeit präsentiert werden, tragen zum Verständnis von Infektionskrankheiten die durch Impfungen verhindert werden können bei, liefern Informationen zu den Impfbemühungen in Laos und dokumentieren die Fortschritte, welche in den letzten Jahren im Land gemacht wurden. Auf der Basis der Ergebnisse dieser Studien wurden Empfehlungen für das Impfprogramm in Laos formuliert, die in mehreren Policy Briefings den lokalen Gesundheitsbehörden mitgeteilt wurden.

LIST OF ABBREVIATIONS

AIC	Akaike Information Criterion
ANC	Antenatal care
anti-HBc	Anti-Hepatitis B core antigen antibodies
anti-HBs	Anti-Hepatitis B surface antigen antibodies
AUC	Area under the curve
BCG	Bacillus Calmette-Guerin
CDC	Centers for Disease Control and Prevention
CdtB	Cytolethal distending toxin subunit B homolog
CH	Central hospital
CI	Confidence intervals
DH	District hospital
DHIS2	District Health Information System 2
DHO	District health office
DTP	Diphtheria-Tetanus-Pertussis vaccine
DTPw-HepB	Diphtheria-Tetanus-Pertussis-Hepatitis vaccine
DTPw-HepB-Hib	Diphtheria-Tetanus-Pertussis-Hepatitis B- *Haemophilus influenzae* type b vaccine
ELISA	Enzyme-linked immunosorbent assays
EPI	Expanded Program on Immunization
EU	ELISA units
GAM	Generalized additive model
GAVI	Gavi, the Vaccine Alliance
GLM	Generalized linear model
HBcAg	Hepatitis B core antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HC	Health centre
HCF	Health care facility
Hib	*Haemophilus influenzae* type b
HlyE	Hemolysin E
HPV	Human papilloma virus
HR	Hospital records
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPL	Institut Pasteur du Laos
IPV	Inactivated polio vaccine
IU	International units
JEV	Japanese Encephalitis vaccine
Lao PDR	Lao People's Democratic Republic
LaoLuxLab	Lao-Lux Research Laboratory “Vaccine preventable diseases”
LIH	Luxembourg Institute of Health
LPRP	Lao People’s Revolutionary Party
LSB	Lao Statistics Bureau
MCV1	First dose of measles-containing vaccine
MDG	Millennium Development Goals
MoH	Ministry of Health

MR	Measles-rubella vaccine
MTCT	Mother-to-child transmission
NCLE	National Centre for Laboratory and Epidemiology (in the Lao PDR)
NIP	National Immunization Program
NITAG	National Immunization Advisory Group
OD	Optical density
OPV	Oral Polio Vaccine
OR	Odds ratio
PaReCIDS	Lao Luxembourg Partnership for Research and Capacity Building in Infectious Disease Surveillance
PBS	Phosphate Buffered Saline
PCV	Pneumoccocal conjugate vaccine
PH	Provincial hospital
PHO	Provincial health office
PRP	Purified polyribosylribitol phosphate capsular polysaccharide of Hib
S. Tyhpi	*Salmonella enterica*, subspecies enterica serovar Typhi
SIA	Supplementary immunisation activities
TCV	Typhoid conjugate vaccines
UHC	Universal health coverage
UNDP	United Nations Development Programme
UNICEF	United Nations Children's Fund
USD	US-Dollars
Vi	Virulence antigen
VIF	Variance Inflation Factor
VPD	Vaccine-preventable diseases
WHO	World Health Organization
YC	(Yellow) Vaccination card

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. Introduction

1.1. The Lao-Luxembourg Friendship Laboratory

The government of Luxembourg has a long-standing relationship with the Lao People's Democratic Republic (PDR). A variety of different projects are carried out in order to provide technical development aid and build capacity to support the country's effort to become increasingly self-dependent and to reach the Millennium Development Goals (MDG). One component of this partnership was to provide support for laboratory surveillance of infectious diseases. Luxembourg was invited by the Ministry of Health of the Lao PDR (MoH) to join French and Japanese public donors to set-up the Institut Pasteur du Laos (IPL) in Vientiane. The IPL was created by Prime Ministerial Decree in November 2007 and started operations in December 2011. In 2012, the Luxembourg Institute of Health (former Institute of Immunology; LIH) set up the Lao-Luxembourg Friendship Laboratory "Vaccine preventable diseases" (LaoLuxLab) at the IPL.

This doctoral thesis describes my serological and epidemiological investigations of vaccine-preventable diseases (VPD) and their implications for vaccination policy in the Lao PDR. My research was carried out within the framework of the project "Luxembourg Lao Partnership for Research and Capacity Building in Infectious Disease Surveillance (PaReCIDS)" with the objective to provide guidance for sustainable capacity in infectious disease surveillance in the Lao PDR. The research work described in this thesis is presented in a series of six scientific articles and one literature review (see section **1.8**). Three of the scientific articles are already published and the remaining four manuscripts are under preparation for the submission to peer-reviewed scientific journals.

1.2. Lao People´s Democratic Republic: A country in transition

1.2.1. Historical context

The founding of the kingdom Lan Xan Hom Khao (*trsl. the kingdom of the "Million Elephants and White Parasols"*) in 1353 by Chao Fa Ngum is generally considered to be the starting point of the history of Laos. The first Lao kingdom consisted of multiple muangs (semi-independent principalities) that recognized one spiritual and political center and one king as their absolute ruler. In 1707 the kingdom separated into three separate monarchies: Luang Prabang in the North, Vientiane in the Center and Champasack in the South. Until the mid-19th century, they were subject to repeated occupations by neighbouring kingdoms, especially Burma (Myanmar) and Siam (Thailand) [17,27].

In the 19th century, France gained more and more influence in South-East Asia. After having established their presence in Vietnam and Cambodia, French colonial powers began the colonization of Laos in 1893. Laos, with the boundaries of today, was integrated into the colony French-Indochina as "Protectorate Laos" [17,18]

During World War II, Japan occupied Laos briefly; however, after its capitulation, the country announced its independence, which was recognized by France in 1953. After the end of the World War II, decades of civil war between the Royal Lao Government and the communist Pathet Lao, as well as the 2nd Indochina conflict followed. In December 1975, the Laotian communists proclaimed an end to the 600-year-old monarchy and declared the "Lao People's Democratic Republic" [17,23]

1.2.2. Political and administrative context

Established in 1975 by the Lao People's Revolutionary Party (LPRP), the Lao PDR of today is a relatively young nation. The country is one of the five communist one-party states that survived the breakdown of the communist state systems between 1989 and 1991. The country is ruled by the LPRP, the only legal political party. A new constitution was implemented in 1991 with amendments following in 2003 and 2015. As written in the constitution, the National Assembly, the parliament of the Lao PDR, elects the president and vice president and approves presidential appointments of the prime minister and members of the cabinet (Council of Ministers). Members of the National Assembly, the president and ministers serve five-year terms [17].

The Lao PDR is separated into 18 provinces (*trsl. koueng*). The provinces are further separated into 148 districts (*trsl. meuang*) which comprise 8447 villages/communities (*trsl. baan*) as of 2018. Vientiane, situated at the Mekong, is the capital of the Lao PDR. Commonly, the Lao PDR is separated in Northern, Southern and Central provinces (**Figure 1**) [55].

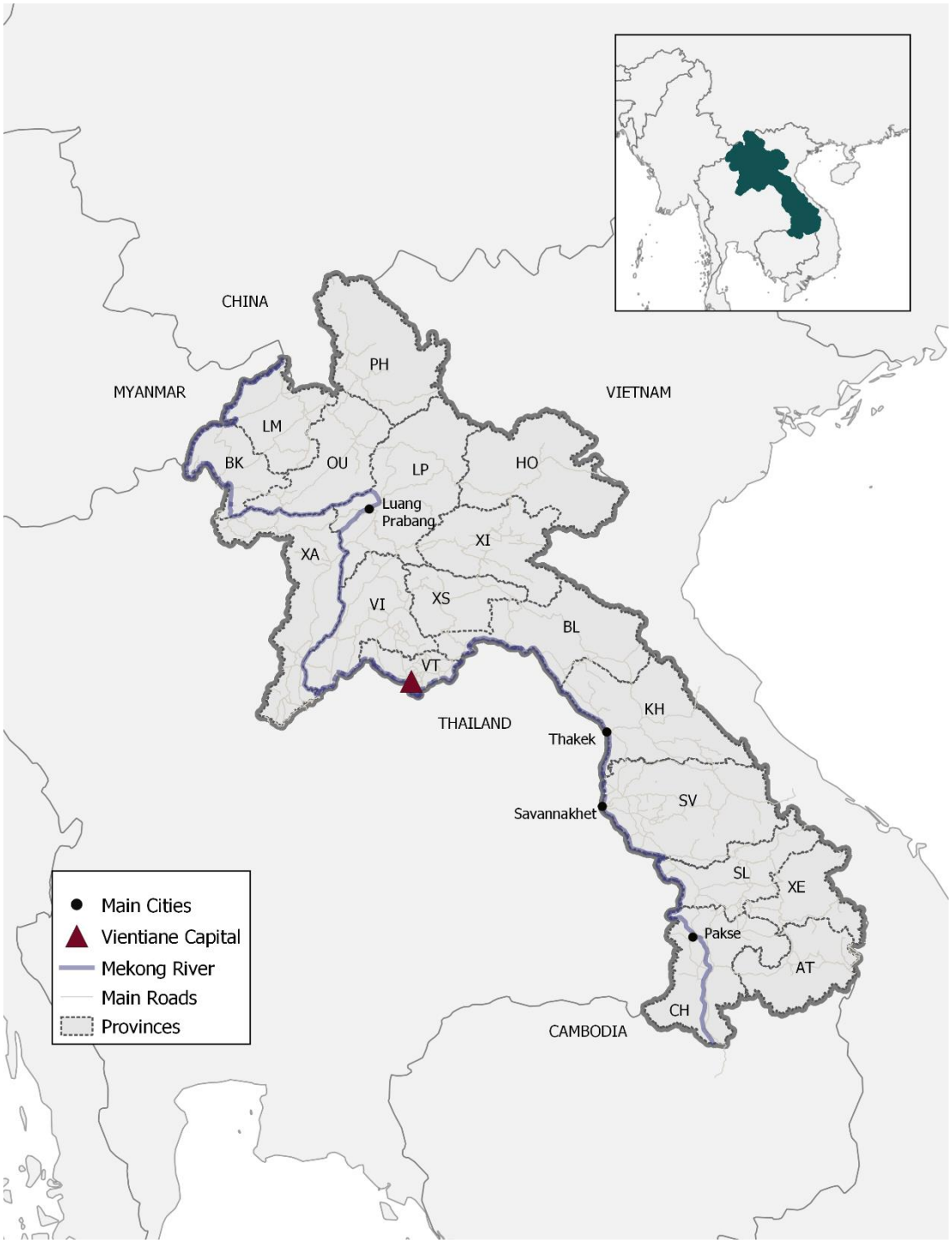

Figure 1 Map of the Lao PDR. AT = Attapeu; BK = Bokèo; BL = Bolikhamxay; CH = Champasak; HO = Houaphan; KH = Khammuan, LP = Luang Prabang; LM = Luang Namtha; OU = Oudomxay; PH = Phongsaly; SL = Salavan; SV = Savannakhét; VI = Vientiane province; VT = Vientiane Capital; XA = Xayabouli; XS = Xaisomboun; XE = Xekong; XI = Xiangkhouang. The map was created with QGIS (QGIS Development Team, 2019) using collected GPS-data and the world borders dataset (http://thematicmapping.org/downloads/world_borders.php, 2019). The data regarding the administrative boundaries of the Lao PDR was obtained from the Humanitarian Data Exchange website (https://data.humdata.org/dataset/lao-admin-boundaries, dataset provided by the National Geographic Department of the Lao PDR, 2019). Projection used: EPSG 4326 – WGS 84.

1.2.3. Geographic, socio-demographic and economic context

The Lao PDR is a landlocked country in South-East Asia, bordering on Myanmar, Cambodia, China, Thailand, and Vietnam (**Figure 1**). The country stretches around 1050 km from Northwest to South-East and is characterized by forest-covered mountains with plains and plateaus in between. The river Mekong represents a natural border to Myanmar and Thailand for a considerable part of the Lao PDR (**Figure 1**). The climate is tropical and influenced by the monsoon pattern. The rainy season generally starts in May and lasts until November, followed by the dry season from December to April [23].

According to the Statistical Yearbook of Lao PDR, the population size was estimated to be 7 million in 2018 [56], but sources vary (**Table 1**). The United Nations Development Programme (UNDP) states the population size with 6.5 million in 2018 on their website [121], while it is roughly 7.2 million on the worldometers information page (an estimate based on UNDP data)[159] and 7.2 million according to the Central Intelligence Agency's The World Factbook [25]. The largest urban centre is the capital Vientiane with 907 000 inhabitants [56]. In total, around 36% of the population live in urban settings [25].

Lao PDR is an ethnically and linguistically diverse country (**Table 1**). The ethnolinguistic fractionalization index, a measure for the probability of two randomly selected individuals belonging to different groups, was estimated to be 0.514 [2]. The population of the Lao PDR can be grouped into four ethno-linguistic families: Lao-Tai (also "Tai-Kadai", former Lao Lum) (66.3%), Mon-Khmer (former Lao Theung) (22.4%), Hmong-Mien (former Lao Sung) (7.5%) and Chinese-Tibetan (2.8%). The total number of ethnic groups is estimated to exceed 200 but only 49 groups are officially recognized by the Lao government [34,48,54]. In addition to Lao, which is the official language, a variety of languages are spoken by ethnic groups [121].

Religious freedom is granted in the constitution of the Lao PDR. Most Lao-Tai are followers of Theravada Buddhism 64.7% [17,54], 1.7% are Christian and 2.1% follow other religions (**Table 1**). About one third (31.5%) state that they do not follow any religion. However, many Lao, especially non-Lao-Tai, follow non-Buddhist folk religions, belonging to animism. Animistic folk beliefs and other religious beliefs are not mutually exclusive and an individual can identify himself for example as both Buddhist and animist. So called spirit houses, a place for those spirits that reside on the land on which the house (or business) was constructed are present at most family homes and buildings [17].

In 2017, the Lao PDR ranked 139 in the Human development index, indicating medium development. The country is currently listed in the least developed country category by the United Nations; however, as of 2018, the country has fulfilled the eligibility criteria to be removed from the list. If the country can sustain its progress, its status could be upgraded as early as 2024 [57].

Table 1 Country characteristics of the Lao PDR.

Country characteristics		Source
Total population	6.5 Million (2018)	[121]
	6.9 Million (2018)	[56]
	7.2 Million (2019)	[25]
	7.2 Million (2020)	[160]
Median age	24.4 years	[160]
Ethnicities	Lao-Tai (66.3%) Mon-Khmer (22.4%) Hmong-Mien (7.5%) Chinese-Tibetan (2.8%)	[54]
Language(s)	Lao (official); Ethnic languages (incl. Hmong and Khmu)	[121]
Gender development index	0.9	[121]
Proportion of seats held by women in the National Assembly	25.50%	[121]
Adult literacy rate	84.70%	[121]
HIV prevalence rate	0.10%	[121]
Life expectancy at birth	66.60 years	[121]
Area	236800 square km	[121]
Poverty rate	23% (2015)	[121]
Human development index	0.586 (2015)	[121]
Gross national income per capita	US$2270 (2017)	[121]
Currency	Kip	
Religions (only institutionalised religions were part of the 2015 census; Buddhism and other non-Buddhist religions are inclusive belief systems in which one person can belong to multiple belief systems)	Buddhism (64.7%) Christianity (1.7%) Other (2.1%) Unspecified/no religion (31.5%) Local non-Buddhist religions (not mutually exclusive with other religions)	[17]

1.2.4. Data quality in the Lao People´s Democratic Republic

Reliable data is essential for evidence-based decision making and for assessing a country's needs. Achieving and maintaining data quality is a general issue which concerns any country or organization; however, lower- or middle-income countries may face different challenges than high-income ones. Official country statistics, such as population size, gender ratio, age distribution and health related and socio-economic indicators are needed for the planning and conducting of epidemiological studies. In this context, it is worth taking a closer look at the situation of data availability in the Lao PDR.

The difficulties in trying to characterize the Lao PDR through numbers is described by Robert Coopers contemporary portrait of the Lao PDR[17]. In his introductory section, he compares the Lao PDR during the time before the latest census in 2015 as a "statistical playground" in which, if "you wanted a particular statistic to support a particular viewpoint, you could probably find it somewhere" (Cooper,

2018, p11-16). Cooper further explains that the reason for this fluctuation of facts was that they arose from estimations that were in constant change rather than having been deliberately falsified and that the situation began to change with the 2015 census that was conducted by the Lao Statistics Bureau (LSB), the national agency for coordinating the collection and dissemination of national statistics[17]. Currently, the statistical capacity indicator of the Lao PDR was estimated to be 64.4 (on a scale from 0 to 100) in 2019 by the world bank in contrast to the regional score of 72.2 for East Asia and Pacific (including high income countries) [109]. In a current research work to assess the quality of official country statistics of countries in the Association of South-East Asian Nations, the Lao PDR was ranked 8th (of ten countries) with a total quality rating of 32.2 on a scale from 10 to 100[107]. Among all South-East Asian countries included, Indonesia ranked highest (total quality rating of 64.2) and Myanmar ranked lowest (total quality rating of 22.9). Indicators relating to data quality that were assessed included for example the professional independence of statistical authorities, impartiality and integrity, sound methodology and procedures and accessibility and clarity. The reasons for the low ratings cited were, amongst others, insufficient separation of official statistics and political interests and the lack of information on statistical methods as well as limited accessibility.

Estimations of certain country parameters, such as population size still vary depending on sources (**Table 1**). The high heterogeneity of the country regarding ethnic groups and geography represents a considerable challenge for data collection.

In the health sector, problems with data quality regarding completeness, accuracy and consistency were reported in a study in the Lao PDR before [112] and are also addressed in one of the articles presented in this thesis (section **CHAPTER 6**). Started in 2014/15, the District Health Information System 2 (DHIS2) is used as an integrative database collecting information regarding several components of the health system such as for example mother and child health, the expanded programme on immunization, and family planning[136]. The system may help capture key health information on a sub-national level in a timely manner. The extent of the benefits from the DHIS2 depends of course on the input received from lower levels of the health care system.

Through efforts such as the implementation of the DHIS2 and providing official country statistics online under the website of the LSB (https://www.lsb.gov.la/en/home/), data quality and availability hopefully continue to improve steadily. At the moment, data in the context of the Lao health system or of VPDs in the Lao PDR are still limited and a large share of information is not distributed through commercial publishing organisations but exists as grey literature.

The research work conducted in the framework of this thesis is based mainly on scientific literature published in peer-reviewed journals, but also as well on grey literature comprising unpublished or not-commercially published reports of governments or organizations, theses, fact sheets, newsletters, working papers or even personal communication (see reference lists).

1.3. The health care delivery system

The health care delivery system in the Lao PDR is widely government-owned. It follows a hierarchical structure with three administrative levels: The MoH oversees the work of the provincial health offices (PHOs) and district health offices (DHOs). Health care services are offered on four levels (**Figure 2**): the central level including central hospitals (CH) in Vientiane Capital, the provincial level, the district level and the health centres (HC) that belong administratively to the district health offices. As of 2017, there are 5 CHs, 17 provincial hospitals (PH), 136 district hospitals (DH) and 1055 HCs. Although the vast majority of health services are delivered through the public system, private health care providers can be found, mainly in urban areas. In 2017, 1028 private clinics and hospitals were listed [1,22,112].

The health situation in the Lao PDR has gradually improved during the past years. The average number of children per woman has decreased from 3.2 in 2011 to 2.7 in 2017 and the proportion of home births has decreased from 59% in 2011 to 35% in 2017 [53,54,117,136]. The life expectancy in 2017 was estimated at 67 years as compared to 49 years in 1980 [62]. Even though the under-five mortality rate has been reduced to 46 deaths per 1000 live births, thereby achieving the MDG 4 (reducing the under-five mortality by two thirds by 2015), it remains the highest in South-East Asia [85,136].

The Lao government aims to achieve universal health coverage (UHC) by 2025. A first step towards UHC was made by introducing the first *Reproductive, Maternal, New-born and Child Health service* package in 2009 and the implementation of a second 10-year strategy starting from 2016 until 2025[136,154].

Despite the progress made regarding key health outcome indicators, there are still considerable gaps and differences in services and outcome of health care between urban and rural areas and between rich and poor families [1].

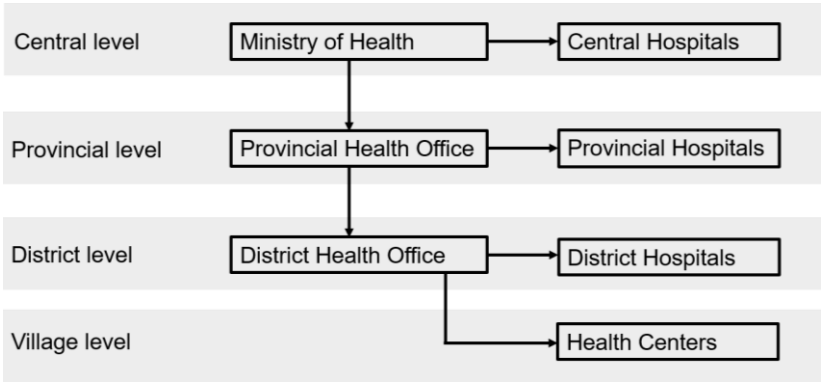

Figure 2 Organization of the health system in the Lao People's Democratic Republic

1.4. Vaccine-preventable diseases addressed in this thesis

Currently, the national immunization programme (NIP) in the Lao PDR (further described in section **1.5**) includes vaccines against tuberculosis, measles, rubella, diphtheria, tetanus, pertussis, hepatitis B, *H. influenzae* type b (Hib), polio, pneumococcal disease and Japanese encephalitis. In the Lao PDR, VPDs still pose a significant public health threat. Outbreaks of VPDs such as diphtheria[74], pertussis[60], typhoid fever[98,119], measles[103] and others have occurred in recent years, despite improvements made in health care and prevention. Research studies offer only an incomplete overview over outbreaks of VPDs or incidence rates in the Lao PDR.

1.4.1. Diphtheria

The World Health Organization (WHO) vaccine-preventable surveillance system (WHO global summary) provides a world-wide summary of reported cases of selected VPDs starting from 1980 to 2018, but does not cover all the years before the year 2000. The numbers of annual reported diphtheria cases in the Lao PDR were below 100, except in the years 2003 (116 cases), 2012 (130 cases) and 2015 (194 cases) (**Figure 3**) [151]. In 2012, the National Centre for Laboratory and Epidemiology (NCLE) in the Lao PDR reported a diphtheria outbreak in two districts in Huaphan province [74].

A study published in 2016 identified cases of the outbreak in 2012 by describing clinical cases, conducting a case-control study and reviewing Diphtheria-Tetanus-Pertussis vaccine (DTP) coverage rates [102]. The study reported that in addition to Huaphan, cases were also reported from six other provinces, whereby most cases were indeed from Huaphan. The vaccination coverage with DTP was suboptimal in Huaphan during the years 2009 to 2012 and reached only 67% in 2012. In the case-control study, nearly half of both controls and cases did not receive any DTP vaccination, only 25% and 12% respectively received the full course.

Another study described the results of a serosurvey in Huaphan province in those districts with most reported cases during the outbreak in 2012[74]. The study found that the anti-diphtheria antibody prevalence was higher among children who lived in villages within a 100 minutes travel time from the nearest health care facility. The travel time to the health care facility, being of Lao Theung ethnicity and having not received advice on immunization at birth were independently associated with non-immunization, while the mother's educational level was a protective factor. Both studies highlighted challenges faced by the NIP in the Lao PDR: Vaccination coverage rates and vaccine immunogenicity may be low in remote areas which are difficult to access.

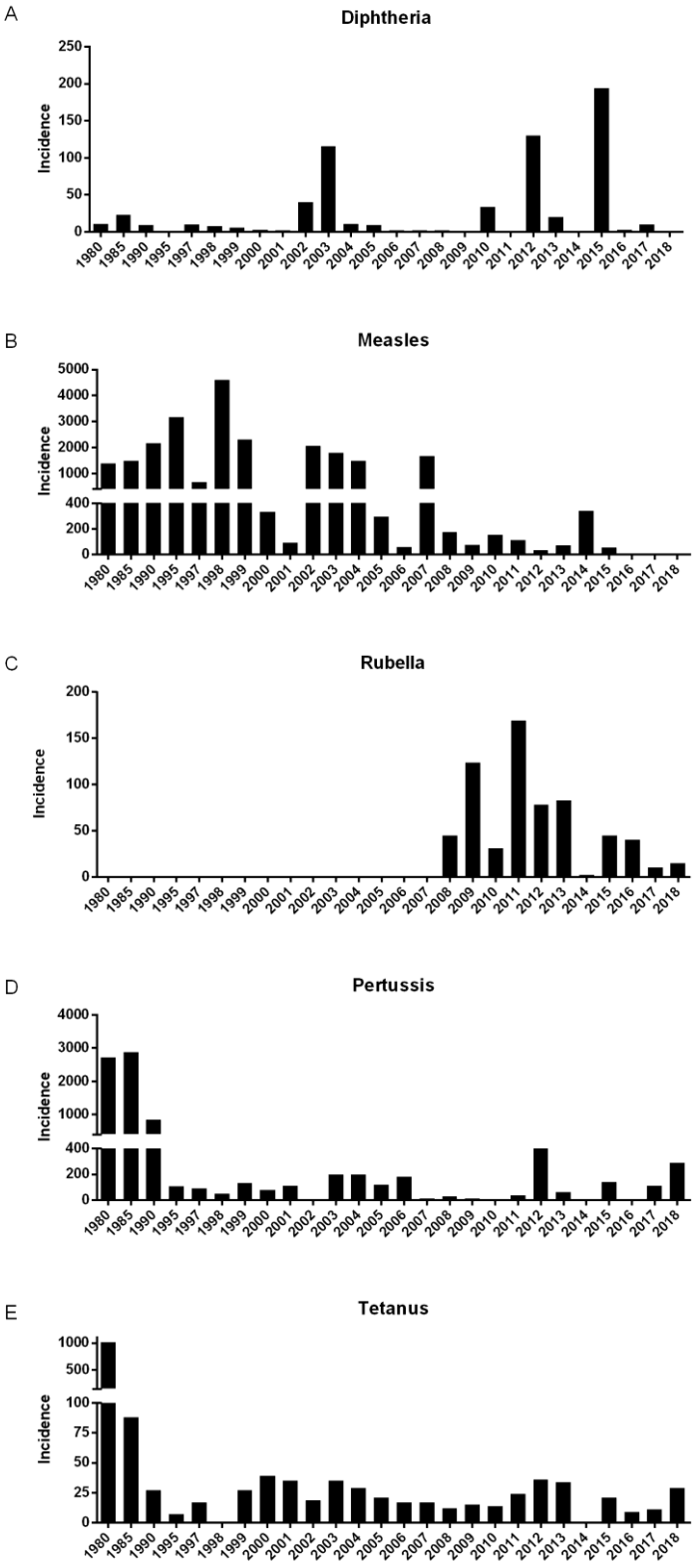

Figure 3 Incidence time series of selected vaccine-preventable disease in the Lao PDR. Incidence time series of Diphtheria (A), Measles (B), Rubella (C), Pertussis (D) and Tetanus (E) in the Lao PDR from 1980 to 2018 as reported to the WHO vaccine-preventable diseases monitoring system (Data obtained from https://apps.who.int/immunization_monitoring/globalsummary/) [151].

1.4.2. *Haemophilus influenzae* serotype b

At the commencement of the research works presented in the thesis in 2016, there were no data on the level of immunity against Hib or the burden of disease in the general population in the Lao PDR. In the meantime, data has been generated in this thesis and others [52]. Section **CHAPTER 3** and **CHAPTER 4** in this thesis describe two research studies investigating the antibody prevalence against Hib.

1.4.3. Hepatitis B

Hepatitis B is endemic in the Lao PDR with 8–10% of the adult population being chronically infected and more than one third of the population having been infected with hepatitis B virus (HBV) at one point in their lives [9,43,44]. The high prevalence of HBV infections in Lao adults also contributes to the high levels of liver cancer in the country (22.4 per 100,000; 5th highest worldwide)[29]. Even though hepatitis B is recognized as a major public health threat, in the absence of a robust surveillance system, it is difficult to obtain a clear understanding of the current situation of HBV epidemiology in the Lao PDR. A systematic review was conducted as part of this thesis in order to provide a comprehensive overview over research studies in the Lao PDR (see section **CHAPTER 6**).

1.4.4. Measles and Rubella

Even though measles vaccination was one of the original vaccines with which the Expanded Program on Immunization (EPI) in the Lao PDR started in 1979[91], measles remained an important public health problem in the country and subject to several studies. As evident from the WHO summary for the Lao PDR, the number of reported cases often exceeded 1000 cases (**Figure 3**). Outbreaks of measles in the Lao PDR have been reported in 2012, 2013, 2014 and 2019 [11,12]. Rubella vaccination was introduced into Lao PDR in 2012, starting with a supplementary immunization campaign[36]. The reported number of cases for rubella in Lao PDR exceeded 100 in 2009 and 2011, but remained below 100 in the rest of the reported years (**Figure 3**). A study focusing originally on varicella zoster virus seroprevalence in the Lao PDR found relatively high numbers of unnoticed measles and rubella cases, suggesting considerable underreporting or other issues with disease surveillance[79].

Data regarding vaccine-induced antibodies against measles and rubella after routine childhood information in the Lao PDR are limited. Protection rates, timeliness of vaccination with the measles-rubella vaccine (MR) and factors associated with seroprotection after routine childhood vaccination are further addressed in this thesis in **CHAPTER 5**.

1.4.5. Pertussis

Pertussis outbreaks occurred frequently during the past years and concerned several provinces [50]. 289 cases were reported to the WHO global VPD surveillance database (**Figure 3**). A seroprevalence study by the LaoLuxLab published in 2020 investigated the prevalence of antibodies in several existing serum

cohorts[50]. The study reported a seroprevalence of 61.6% in adults born before the introduction of pertussis vaccination in the Lao PDR, indicating exposure to *Bordetella pertussis*. Almost half of the health care workers were found to be negative for anti-*B. pertussis* antibodies and therefore being unprotected. Another concerning finding was the relatively low seroprevalence rate in vaccinated children under five years of age (61.8%)[50].

Even though numerous pertussis outbreaks were documented in the past years, data on current anti-pertussis antibody prevalence after routine childhood vaccination are scarce. Vaccine-induced immunity against *B. pertussis* is discussed in **CHAPTER 4** of this thesis.

1.4.6. Tetanus

Tetanus vaccination during antenatal care (ANC) is an essential intervention that protects the mother and new-born against fatal maternal and neonatal tetanus[115]. Tetanus vaccination for children was included in the NIP in the Lao PDR since the beginning[91].

Cases of neonatal tetanus reported to the WHO global VPD surveillance summary remained well below 50 for the years reported (spanning 1990 to 2018). Numbers of all tetanus cases also did not exceed 50, except in the years 1980 and 1985 (**Figure 3**)[151]. Maternal and neonatal tetanus elimination was achieved in the Lao PDR in 2014 [84].

A recent study in three districts of Savannakhet province in the South of Lao PDR showed that maternal tetanus vaccination during pregnancy was inadequate, as only 77% of the mothers and 79% of their new-borns had anti-tetanus antibody titers indicative of protection. Furthermore, the coverage rates of mothers with tetanus toxoid was also insufficient[84].

CHAPTER 4 in this thesis describes a study investigating the levels of vaccine-derived immunity against tetanus in the context of the pentavalent vaccine.

1.4.7. Typhoid fever

Typhoid fever is a notifiable disease in the Lao PDR and cases are reported to the NCLE in Vientiane. However, access to health care is still inadequate especially in remote areas and the capacity for blood culture testing in the Lao PDR is limited to only three laboratories in the whole country. Currently, the requirements for an adequate typhoid fever surveillance are not met which complicates an assessment of disease burden in the Lao PDR [116]. The lack of data also impeded recent considerations of including a typhoid fever vaccine into the national immunization schedule. **CHAPTER 2** of this research work is focusing further on typhoid fever in the Lao PDR.

1.5. Expanded Program on Immunization

Despite the improvements made in public health in the Lao PDR, the incidence of VPDs is high and vaccination remains one of the most important health interventions[14,74,164]. The EPI that was established by the WHO in 1974 with the goal to provide vaccination services to all children worldwide [149], was launched in the Lao PDR in 1979. At the beginning, six vaccines were included in the NIP: the Bacillus Calmette-Guerin (BCG) vaccine and vaccines against diphtheria, tetanus, pertussis, polio, and measles. This initial vaccination schedule was expanded step-by-step to include first a hepatitis B vaccination (in 2001), the hepatitis B birth dose (in 2003)[91], Rubella vaccination (2012)[36], Pneumococcal conjugate vaccine (PCV) (2013)[101], Japanese Encephalitis (JEV) (2015)[128] and Human papilloma virus (HPV) (2013)[59]. By replacing the Diphtheria-Tetanus-Pertussis-Hepatitis B vaccine (DTP-HepB) with the pentavalent Diphtheria-Tetanus-Pertussis (whole cell)–Hepatitis B–*Haemophilus influenzae* type B (DTPw-HepB-Hib) vaccine, Hib vaccination was introduced in the Lao PDR in 2009 [91]. The introduction of the inactivated polio vaccine (IPV) in addition to the routinely used oral polio vaccine (OPV) was implemented in the last few years[90]. **Figure 4** shows the current format of the yellow vaccination card used in Lao PDR.

Generally considered as one of the most successful interventions in the public health sector in the Lao PDR, the EPI achieved poliomyelitis elimination in 2000[137] and neonatal tetanus elimination in 2014[144]. Even though the coverage rates steadily progressed between 2009 and 2017, the national goal of achieving 90% vaccination coverage in 2015 was not met[91,136].

The progress made by the EPI remains uneven, leaving many areas at risk. Outbreaks of pertussis, measles and diphtheria have been reported in recent years in all regions of the country [75,91,124,141] and suggest pockets of suboptimal vaccination coverage. Indeed, even though vaccination coverage rates have improved considerable throughout the past years, variation in coverage rates differs widely among provinces and districts. The overall full immunization coverage (BCG, Polio dose 3, DTP-Hib-HepB dose 3 and MR) in children below 1 year of age increased from 27.7% in 2015 to 77% in 2019. As per the immunization schedule in the Lao PDR, all vaccines should be given in the first year of life, except for the second dose of measles vaccine. However, since the hepatitis B birth dose, the inactivated polio vaccine (IPV), PCV and JEV are not universally administered within the country, they are usually excluded from estimating the full immunization coverage. In 2019, Phongsaly province in the North of Lao PDR reported the lowest proportion of fully vaccinated children under 1 year with 28.2% as compared to Champasack with 98% [22]. The heterogeneity of vaccination coverage rates between areas becomes even more evident by looking at the individual districts. **Figure 5** shows the full immunization coverage rates in children under 1 year of age by districts, based on data provided in the DHIS2 database in the years 2017, 2018 and 2019. Even though the hepatitis B birth dose was introduced into the immunization schedule in 2003, coverage rates remain low in most districts **Figure 5**. Vaccination

coverage with DTP-HepB-Hib was estimated at 84% in 2018 and even though coverage rates varied greatly in the years 2017, 2018 and 2019, an increasing trend in coverage rates is visible **Figure 6**). The overall national coverage rates for the first dose of measles-containing vaccine (MCV1) increased from 40% in 2007 to 87% in 2014 as reported by both WHO and official country data estimates. Since then, coverage rates with MCV1 ranged between 83% and 69% as reported by WHO estimations and 88% to 76%, according to the official country estimates [157]. Looking at the district data (**Figure 6**), there seems to be an increasing trend in districts achieving a coverage exceeding 95% through the years 2017 to 2019.

In order to support the vaccine decision-making processes in the country, a National Immunization Advisory Group (NITAG) was established in the Lao PDR in 2013 [80]. The NITAG provides country-specific recommendations on which vaccines to include in the program, the schedule and other vaccine-related issues.

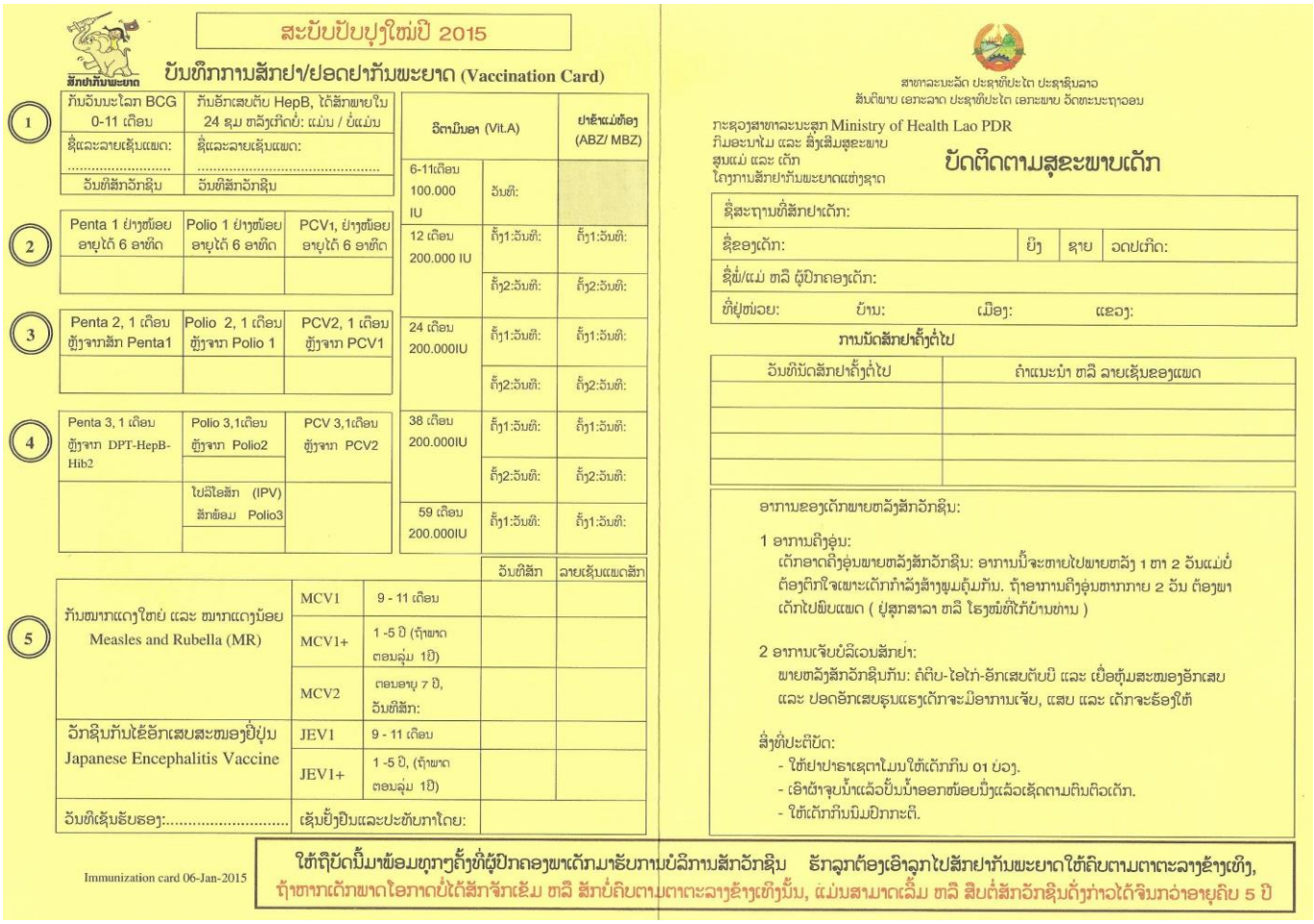

Figure 4 Immunization card used in the Lao People´s Democratic Republic.

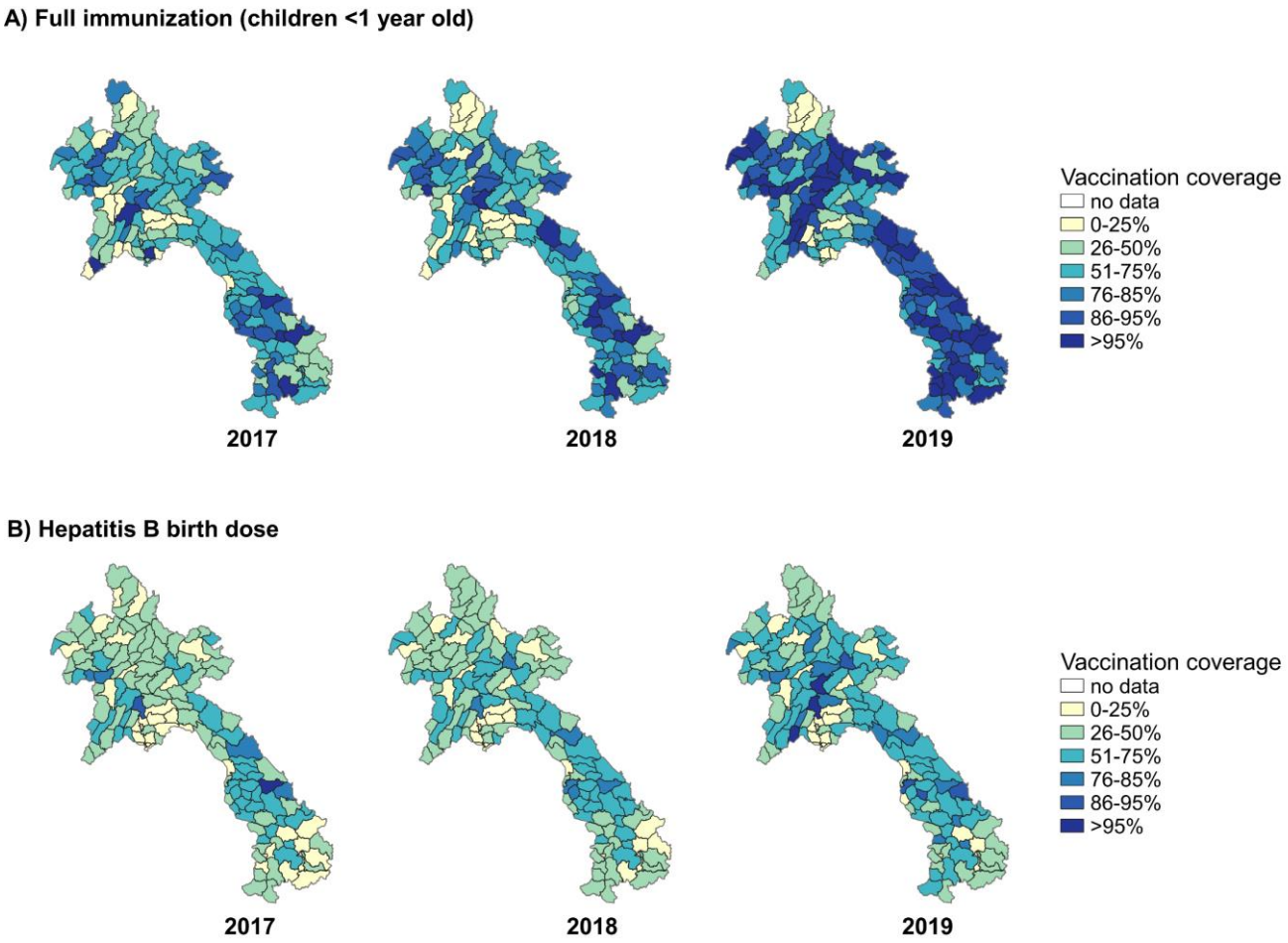

Figure 5 Vaccination coverage rates in the Lao PDR by districts. **A** Full immunization coverage of children under 1 year of age in 2017-2019 by district. **B** Vaccination coverage with hepatitis b birth dose in 2017 to 2019 by district. The map was created with QGIS (QGIS Development Team, 2019) using collected GPS-data and the world borders dataset (http://thematicmapping.org/downloads/world_borders.php, 2019). The data regarding the administrative boundaries of Lao PDR was obtained from the Humanitarian Data Exchange website (https://data.humdata.org/dataset/lao-admin-boundaries, dataset provided by the National Geographic Department of the Lao PDR, 2019). Projection used: EPSG 4326 – WGS 84. Vaccination coverage data was obtained from the District Health Information Software (DHIS2). Data was accessed: 12.04.2020 (http://hmis.gov.la/).

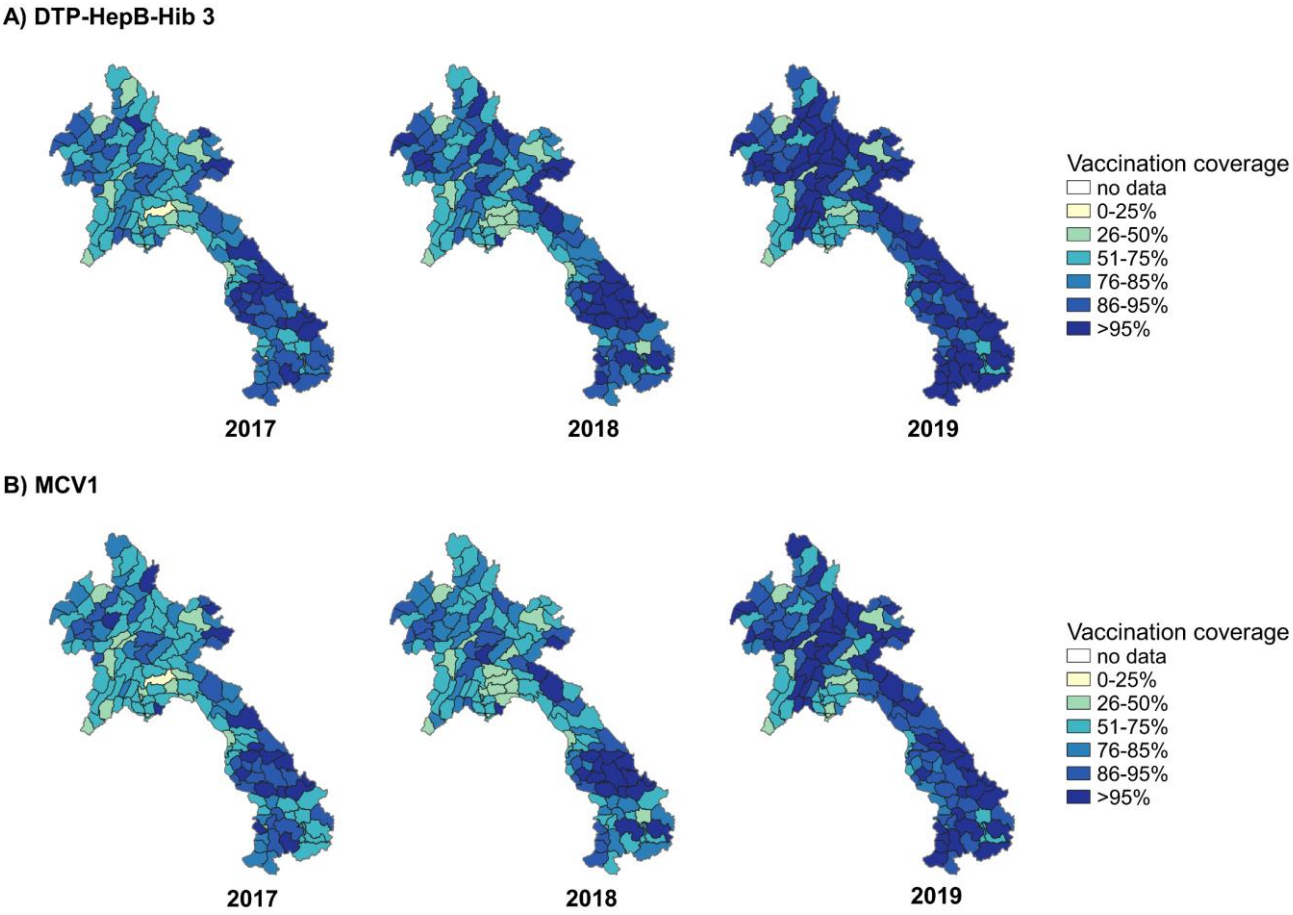

Figure 6 Vaccination coverage rates in the Lao PDR by districts. **A** Vaccination coverage with DTP-HepB-Hib 3 in 2017-2019 by district. **B** Vaccination coverage with MCV1 in 2017 to 2019 by district. DTP-HepB-Hib = third dose of the pentavalent diphtheria-tetanus-pertussis–Hepatitis B–Haemophilus influenzae type B vaccine (DTPw-HepB-Hib) vaccine. MCV1 = Measles-containing-vaccine first-dose. The map was created with QGIS (QGIS Development Team, 2019) using collected GPS-data and the world borders dataset (http://thematicmapping.org/downloads/world_borders.php, 2019). The data regarding the administrative boundaries of the Lao PDR was obtained from the Humanitarian Data Exchange website (https://data.humdata.org/dataset/lao-admin-boundaries, dataset provided by the National Geographic Department of the Lao PDR, 2019). Projection used: EPSG 4326 – WGS 84. Vaccination coverage data was obtained from the District Health Information Software (DHIS2). Data was accessed: 12.04.2020 (http://hmis.gov.la/)

1.6. Vaccine management

The distribution of vaccines follows the administrative line of the health care system as described above (section **1.3**). Upon arrival in the country, the vaccines are stored in a central storage facility in Vientiane, from where the facilities of the capital and the provincial levels are supplied. The PHO supervises the distribution of vaccines to the DHs from where connected HCs are supplied. Immunization services are offered either directly at the facilities or through outreach services[49,141].

In the past, the NIP has largely relied on funding through international organizations including WHO, United Nations Children's Fund (UNICEF), Centers for Disease Control and Prevention (CDC) and Gavi, the Vaccine Alliance (GAVI). Between 2010 and 2016, the overall spending on immunization services increased from 4.3 million USD to 24.8 million USD, mainly through support from GAVI. However, as the country's income exceeded the eligibility threshold for GAVI support, it moved into the accelerated transition phase in 2017. At the end of the 5 year transition phase, the government is expected to be fully self-financing its vaccination program [135].

1.7. The role of seroepidemiology in the surveillance of vaccine-preventable diseases

The surveillance of VPDs is essential for public health efforts in order to assess the burden of disease and the level of protection in the population, before and after vaccination has been introduced. The WHO "Overview of VPD Surveillance Principles" [143] clearly state the specific objectives of VPD surveillance:"

1. Monitoring disease elimination or eradication effort;
2. Detection of outbreaks and new pathogen;
3. Evidence for new vaccine introduction or optimizing vaccine schedules;
4. Evaluation of immunization program performance and defining the need for supplementary immunization;
5. Vaccine effectiveness, impact on disease burden, or both;
6. Changes in disease strains or type" (WHO, 2018, p6)

Information regarding mortality and morbidity rates represent one way in monitoring VPDs in order to investigate disease dynamics and the impact of vaccination. However, these estimates cannot provide information regarding the level of immunity in the population [72].

Serology is the study of proteins, especially antibodies, found in blood or other bodily fluids. A variety of serological methods including agglutination, immunoblotting, plaque neutralization test, chemiluminescent techniques and Enzyme-linked immunosorbent assay (ELISA), can be used to identify antibodies and antigens, depending on the research objectives.

Seroepidemiology, in turn, is defined as "the systematic collection and testing of blood samples from a target population, or a representative sample thereof, to identify current and past experiences with infectious diseases by means of antibody and antigen tests and by measurement of other indices of immunity" (Stanberry, 2014, p68) [108]. Since antibody responses can either be a result of natural infection with the pathogen or elicited through vaccination, seroepidemiology is utilized for the surveillance of VPDs. Serological data provides insights into the disease epidemiology before and after a vaccine has been introduced and about the impact of the vaccine [19].

Using serological data to complement our understanding of VPDs has many advantages: Antibodies are long-lasting and may be present even after an infection has been cleared, subclinical infections can be detected, in absence of a working reporting system, antibodies could act as biomarkers and potentially vulnerable and risk groups can be identified. On the other hand, there are several drawbacks of the utilization of serological data: natural and induced immunity can often not be distinguished, which complicates the interpretation of the results, the necessary capacities for performing the laboratory work and data analysis are needed and even though antibodies are long-lasting, they may wane [19,72,132].

Prior to the introduction of a vaccine, seroepidemiology can aide in assessing the burden of disease and herd immunity in the population. After a vaccine has been introduced, seroepidemiological data can be used to determine the duration of the conferred protection, identify gaps in population immunity, evaluate the impact of vaccination campaigns and estimate effective coverage rates [19].

It has been suggested to include serological data as part of the routine surveillance systems for VPDs [132]. In some countries, serological surveillance is already in place (i.e. The European Sero-Epidmeiology Network [3]) and serology has been used to assess progress in measles and rubella elimination and poliomyelitis eradication [19]. In Vietnam, concrete plans for a nationwide serosurveillance system have already been developed [6].

1.8. Research projects included in this thesis

1.8.1. Research objectives

This doctoral thesis describes my serological and epidemiological investigations of VPDs and their implications for vaccination policy in the Lao PDR. The overall objectives of this research work are (i) to characterize of the epidemiology of VPDs for which there is no routine vaccination in the Lao PDR, (ii) to assess the level of protection against VPDs and investigate possible risk factors or predictors for seroprotection and (iii) to evaluate the impact of vaccination.

These overall objectives were broken down into several separate research studies, based on the specific needs and knowledge gaps in the country. The following questions were addressed in the seven scientific articles presented in this thesis:

1. The epidemiology of VPDs before the introduction of vaccination or for which there is no routine vaccination in the country
   - What is the prevalence of antibodies against Salmonella enterica, subspecies enterica serovar Typhi (*S.* Typhi) in the general population of the Lao PDR?
   - What were the levels of natural and vaccine-derived immunity against *H. influenzae* type b before and after the introduction of the vaccination?

2. Assessment of the level of seroprotection against VPDs and investigation of possible risk factors for seroprotection
    - Is there an improvement of seroprotection/seropositivity rates induced by the pentavalent Diphtheria-Tetanus-Pertussis (whole cell) –Hepatitis B–*Haemophilus influenzae* type B or measles-rubella vaccination in 2017 after improvements in vaccine management since 2013/14?
    - Are there predictors/risk factors associated with seroprotection through vaccination with the DTPw-HepB-Hib or MR vaccines?
    - What is the timeliness of vaccination with DTPw-HepB-Hib and MR?
3. Evaluation of the impact of vaccination
    - What is the current understanding of hepatitis B epidemiology in the Lao PDR?
    - What was the long-term impact of hepatitis B vaccination 17 years after its introduction in the Lao PDR?

1.8.2. Thesis structure

This thesis is compiled in cumulative form, comprising six research articles and one review article in their published or latest versions. Three of the manuscripts have been published already; the others are currently under preparation. The cumulative form of the thesis necessitates a certain repetition in article introduction and methods sections, but it otherwise describes the specific objectives addressed in each article.

The thesis consists of a general introduction in **CHAPTER 1** providing the necessary context and background for the research articles described in this thesis, followed by the research articles, each corresponding to one chapter, and one overall discussion chapter. **CHAPTER 2** and **CHAPTER 3** address the research questions regarding epidemiology of VPDs before the introduction of vaccination or for which there is no routine vaccination in the country; **CHAPTER 4**, **CHAPTER 5** and **CHAPTER 6** investigate the levels of seroprotection against selected VPDs, the timeliness of the vaccination and possible risk factors or predictors for seroprotection and **CHAPTER 7** and **CHAPTER 8** look at the research questions regarding hepatitis B. Each paper-format chapter includes a bridging section describing the rationale for the study and its place in the narrative of this thesis. **CHAPTER 9** comprises a discussion and conclusion section connecting the presented research work.

A list of manuscripts (published and unpublished) and conference presentations can be found in the Appendix **A** and **B** respectively.

CHAPTER 2. An age-stratified serosurvey against purified Salmonella enterica serovar Typhi antigens in the Lao People´s Democratic Republic

Lisa Hefele[1,2], Antony P. Black[2], Trinh Van Tan[3], Nguyen Minh Tri [4,5], Nguyen Duc Hoang [4,5], Siriphone Virachith [2], Claude P Muller[1], Judith M. Hübschen[1], Paula Russell[6,7], Josefin Bartholdson Scott[6,7], Chau Nguyen Ngoc Minh[3], Tran Vu Thieu Nga[3], and Stephen Baker[6,7]

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[7]Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge Biomedical Campus, Cambridge, United Kingdom

2.1. Contribution statement

The results presented in this study were gained from a collaborative work and the research manuscript presented here is currently under preparation for submission to a peer-reviewed journal. The study was conceived by Dr Black, Dr Baker and Ms Hefele. Laboratory work was conducted by Ms Hefele, with the assistance of Dr Virachit and Dr Tan, based on a protocol provided by Dr Baker and Dr Tan. Data analysis of the serological data were conducted by Ms Hefele with assistance from Dr Tan and Dr Baker. The initial draft of the manuscript was written by Ms Hefele. All authors wrote and commented on the manuscript. The supplementary material can be found in Appendix **C.a.**

2.2. Bridging section

In the first study presented in this thesis, serological data is used to characterize *S.* Typhi epidemiology in Lao PDR. At present, typhoid fever is not one of the VPDs targeted by the NIP and little is known about the epidemiology of typhoid fever in the Lao PDR, even though outbreaks occur frequently [98,119]. Typhoid fever is a notifiable disease in the Lao PDR, but disease surveillance is hampered due to the fact, that the capacity to conduct blood culture is limited to three health care facilities in the country. The MoH of the Lao PDR expressed an interest in a typhoid fever burden assessment to inform on the question whether to introduce typhoid fever vaccination in the country or not.

The decision on introducing a vaccine is based on numerous issues, ranging from the determination of disease burden, public perception, safety and efficacy considerations and cost-effectiveness to vaccine management issues such as supply chain and the previous performance and capacity of the NIP. Serological data can help investigating the disease burden in settings where clinical data is lacking and/or underreported. In addition, serological evidence on disease burden with respect to age groups, spatial differences or other influential factors could help with deciding how to utilize resources in the most efficient and timely manner.

Given the challenging nature of infectious disease surveillance in the Lao PDR, there clearly is a need for more data on disease incidence and prevalence. The objective of the study presented here was to investigate the prevalence of three anti-*S.* Typhi antibody markers in randomly selected samples from existing serum cohorts collected from different age groups and locations in order to establish baseline data for the design of follow-up studies.

2.3. Abstract

Introduction

The epidemiology of typhoid fever in Lao People`s Democratic Republic (PDR) is poorly defined. Estimating the burden of typhoid fever in endemic countries is complex due to the cost and limitations of population-based surveillance; serological approaches may be a more cost-effective alternative.

Methods

ELISAs were performed on 937 serum samples (317 children and 620 adults) from across Lao PDR to measure IgG antibody titers against Vi polysaccharide and the experimental protein antigens, CdtB and HlyE. We measured the significance of the differences between antibody titers in adults and children and fitted models to assess the relationship between age and antibody titers.

Results

There was a significant difference in median anti-HlyE IgG titer between children (351.7 ELISA Units (EU)) and adults (198.1 EU; $p<0.0001$). Similarly, the median anti-CdtB IgG titer was significantly higher in children than adults (52.6 vs 12.9 EU; $p<0.0001$). Conversely, the median anti-Vi IgG titer was significantly higher in adults than children (11.3 vs 3.0 U/ml; $p<0.0001$). A non-linear trend line fitted to the anti-CdtB and anti-HlyE IgG data identified a peak in antibody concentration in children <5 years of age. The anti-HlyE IgG and anti-CdtB IgG titers correlated reasonably well with each other in both adults and children. The anti-Vi IgG data did not correlate with either of the other two antibody profiles.

Discussion

We identified elevated titers of anti-HlyE and anti-CdtB IgG in the serum of children residing in the Lao PDR. These antigens are associated with seroconversion after typhoid fever and may be a superior measure of disease burden than anti-Vi IgG. Evidently, additional data are required to better assess the performance of these serological markers, but this approach is scalable and may be developed to assess the burden of typhoid fever in countries where the disease may be endemic, and evidence is required for the introduction of typhoid vaccines.

2.4. Manuscript

Introduction

Typhoid fever is a systemic disease caused by the bacterium *Salmonella enterica*, subspecies enterica serovar Typhi (*S.* Typhi) and is transmitted through contaminated food or water. Between 128000 to 161000 deaths are attributed to the disease per year [146]. Diagnosis of typhoid fever by blood culture is considered to be the gold standard, but poses a challenge for many countries [8,88,95,153]. As an

alternative, the serology-based Widal test is another diagnostic method, but its utilization remains controversial [82,88]. Due to the inadequate performance of the currently used diagnostic tests, the search for novel methods is on-going [20,118].

The investigation of serological markers for typhoid fever prevalence may aid overcoming diagnostic challenges and provides estimates of subclinical typhoid fever infections. Exposure rates among the general population are not routinely evaluated by cross-sectional studies that utilize *S.* Typhi antigens; however, in certain studies, serology-based assays were used to measure seroprevalence rates [42,120,126]. Antbodies against hemolysin E (anti-HlyE) and cytolethal distending toxin subunit B homolog (anti-CdtB) have been previously suggested to be useful biomarkers in distinguishing typhoid fever cases from non-typhoid fever cases [5,13,104,118]. Other studies focus on antibody responses against the immunogenic polysaccharide capsular Vi "virulence" antigen (anti-Vi) which presents the prime target for vaccine development [30,42,126].

In the Lao PDR, outbreaks of typhoid fever [98,119] have occurred in recent years. Typhoid fever is a notifiable disease in the Lao PDR and typhoid fever cases are reported to the NCLE in Vientiane. However, access to health care is still inadequate especially in remote areas and the capacity for blood culture testing in the Lao PDR is limited to only three laboratories in the whole country [116]. Currently, the requirements for an adequate typhoid fever surveillance are not given which hinders an assessment of disease burden in the Lao PDR. The lack of data also impeded recent considerations of including a typhoid fever vaccine into the national immunization schedule.

Recently, a study provided insights into clinical data obtained at a CH in Vientiane Capital over the time period of 18 years[98]. The study reported the annual numbers of typhoid fever patients to decrease since 2010 and estimated the annual in-hospital incidence of typhoid fever in 2018 to be 0.59 per 100 000 people. Another study published also in 2020 [12] estimated that the annual incidence of typhoid fever in Vientiane was relatively low with 4.7 per 100 000 persons for the time period of 2015 to 2017. These two studies are the first ones to focus exclusively on typhoid and paratyphoid fever in the Lao PDR and provide a much needed, first assessment of the situation. Unfortunately, the presented findings are limited by the geographic areas included and the biased nature of the data, thus resulting likely in an underestimation of typhoid fever incidence.

In order to contribute to the understanding of typhoid fever epidemiology in the Lao PDR, we conducted a serological, cross-sectional study using existing serum samples from different age groups and areas of the Lao PDR. This study is the first serology-based study in Lao PDR and provides initial insights into age-related exposure to *S.* Typhi and baseline antibody titers against the HlyE, CdtB and Vi antigen in the general population.

Methods

Ethical approvals

The studies generating samples and data for this study were approved by the Lao National Ethics Committee (Cohort 1: 059/2013/NECHR, 022/2014/NECHR, 033/2017/NECHR, 032/2017/NECHR, 031/2017/NECHR, 056/2017/NECHR, and 038/2016/NECHR; Cohort 2: NECHR 059/2013 and 059/2014)

Study population

The samples for this study originated from two independent cohort studies; Cohort 1 and Cohort 2. Cohort 1 (child cohort) was comprised of 317 children and adolescents aged between 9 months and 15 years. These individuals were selected randomly (with respect to age and sex) from three different cohorts that were collected within a framework of other studies [40,41]. The studies generating these serum samples were conducted in central part of Lao PDR between 2017 and 2018. Two of these studies were cross-sectional seroprevalence studies focusing on VPDs [40,41]. The third study was a hospital-based study focusing on transfusion-transmissible infections in blood transfusion recipients (manuscript in submission). From this study, only the controls were randomly selected for the anti-*S.* Typhi testing. Cohort 2 (adult cohort): was comprised of 620 serum samples of blood donors aged between 17 and 40 years who were recruited in the context of an alternative research study between 2013 and 2015 (manuscript submitted). The samples were randomly selected from a total of 5,018 and stratified by age, sex, and province.

Serological testing

ELISAs were performed on all serum samples to measure IgG (immunoglobulin G) antibody titers against Vi, CdtB, and HlyE.

To determine the quantity of anti-Vi IgG antibodies, a commercial ELISA kit (Vacczyme, Binding site, UK) was employed; assays were conducted according to the guidelines of the manufacturers. Antibody concentrations were derived from the optical density (OD) data using a standardized curve-fitting 4-parameter logistic method. Any samples producing an OD below the calculation limit of the Vacczyme assay was classified as “left-censored” for the purposes of the analysis.

Anti-HlyE IgG and anti-CdtB IgG ELISAs were performed according to a previously described protocol [118]. Briefly, 96 well flat-bottom ELISA plates (NunC 442404, Thermo Scientific) were coated overnight with 100 ml per well of the various antigens (final concentrations; 7 µg/ml of CdtB antigen and 1 µg/ml of HlyE antigen in 50 mM Carbonate Bicarbonate buffer). Coated plates were washed and blocked with 5% milk solution in Phosphate Buffered Saline (PBS) for two hours. After the blocking, plates were washed and incubated with 100µl sample (1:200 dilution) at room temperature. Plates were

incubated with 100µl per well of alkaline phosphatase conjugated anti-human IgG (Sigma) for one hour at room temperature. Plates were developed using p-Nitrophenyl phosphate (SigmaFAST N1891, SigmaeAldrich, UK) substrate for 60 minutes at ambient temperature and the final absorbance was read at dual wavelengths (405 nm and 490 nm) using an automated microplate reader. Antibody concentrations in ELISA units (EU) were derived from the OD data using a standardized curve-fitting 4-parameter logistic method. If the measured antibody concentration was above or below the calculation range, the sample was tested again in a higher or lower dilution. The anti-HlyE IgG titre and anti-CdtB IgG titers in one (0.1%) and 15 (1.6%) samples, respectively, were repeatedly higher than the calculation limits; these samples were given the highest value measured on the same plate for the purpose of the analysis. Two (0.2%) of the samples had anti-CdtB IgG titres that were repeatedly below the calculation limit; these were excluded from the analysis. The results of each ELISA plate were accepted only if the OD values of the quality controls were within the acceptable range.

Data analysis

Anti-Vi IgG data containing left-censored values were analysed using methods described in the NADA package [65]. The left-censored data were stored using an indicator variable: The first variable contained the measured titre data and values below the calculation limit were stored as the lowest limit (7.4 U/ml). The second variable indicated if the value was a true measurement or censored. Summary statistics of the anti-Vi IgG data were calculated using robust regression on order statistics to account for left censoring of the data. Antibody titres measured by ELISA were log transformed. After analysis of normality of independent variables in the different groups (using the Shapiro-Wilk test) and homogeneity of the variances between the groups (using Levene's test), non-parametric statistical tests were employed. Wilcoxon test or Kruskal-Wallis test followed by Dunn's multiple comparison tests with Bonferroni correction were used to test the significance of the differences between the antibody titer measured in groups. In case of the left-censored anti-Vi IgG data, a generalized Wilcoxon test was used ("cendiff", NADA package [65]). The Spearman correlation coefficient or Kendall´s tau (in case of censored data) were calculated to measure the association between antibody levels determined by ELISA.

In order to assess the relationship between age and anti-HlyE IgG and anti-CdtB IgG antibody levels, both a linear regression model and a generalized additive model were fitted to the data. Generalized additive models are regression-based models that estimate non-linear trends for the predictor variable without making assumptions about the shape of the function. The anti-Vi IgG data was fitted as a function of age using Akritas–Theil–Sen non-parametric regression to account for the left-censored data. A p value <0.05 was considered statistically significant. Data analyses were conducted using R statistical software [94] with the following packages: tidyverse [131], ggbeeswarm[15], ggpubr [46], mgcv[133], rstatix[47], fitdistrplus [21], plotrix [61] and NADA[65].

Results

Population characteristics

In total, serum from 937 participants were included in the study and originated from a range of provinces across Lao PDR **Table 2** and Appendix **C.a**, **Supplementary Figure 1**). The majority of children in Cohort 1 were from Vientiane (249/317; 78.6%) and most (171/317; 53.9%) were female. The age of participants in Cohort 1 (children) ranged from 0 to 15 years, with a median age of 8 years. The majority (373/620; 60.2%) of the participants in Cohort 2 (adults) were male (**Table 2**) and over a third (232/620; 37.4%) considered themselves students. The age of participants in Cohort 2 ranged from 17 to 40 years, with a median of 26 years.

Table 2 Characteristics of study participants in adult and child cohorts.

		Children (n=317)	Adults (n=620)
Study year		2017-2018	2013-2015
Age (years)	min - max	0 - 15	17 - 40
	mean	7.56	26.34
	median	8	26
Sex (%)	male	46.06	60.16
	female	53.94	39.84
Province (%)	Attapeu	-	13.23
	Bolikhamxay	21.45	0.00
	Huaphan	-	10.00
	Khammuane	-	17.42
	Luang Namtha	-	11.61
	Luang Prabang	-	16.13
	Vientiane Province & Capital	78.55	15.65
	Phongsaly	-	4.35
	Xayabouli	-	11.61
Profession (%)	office worker	-	28.06
	soldier	-	25.48
	student	-	37.42
	other	-	9.03

The distribution of anti–S. Typhi serum IgG antibodies within the population

We measured IgG antibodies targeting HlyE, CdtB, and Vi antigen in serum from the 937 participants. Overall, the anti-Vi antibody titres ranged from 7.4 U/ml to 600 U/ml, the anti-HlyE IgG antibody titres ranged from 12.6 ELISA units (EU) to 5,163.2 EU, and the anti-CdtB IgG antibody titres ranged from 2.8 to 1,466.1 EU (**Table 3**). Notably, 469/937 (50.1%) of the samples generated anti-Vi IgG titres that were below the calculation limit of 7.4 U/ml. The median anti-HlyE, anti-CdtB, and anti-Vi IgG titres among all participants were 234.3.8 EU, 16.8 EU and 7.5 U/ml, respectively (**Table 3**).

Table 3 Anti–*S.* Typhi serum IgG antibody titers in the adult and child cohorts.

		N	N cens	Median	Mean	sd	Max	Min
anti-HlyE IgG (EU)	All data	937	0	234.32	453.83	672.37	5163.20	12.64
	Cohort 1: Children	317	0	351.74	734.59	980.75	5163.20	28.34
	Cohort 2: Adults	620	0	198.14	310.29	362.72	4520.40	12.64
anti-CdtB IgG (EU)	All data	935[1]	0	16.80	77.15	176.20	1466.10	2.75
	Cohort 1: Children	317	0	52.59	178.68	270.01	1466.10	3.73
	Cohort 2: Adults	618	0	12.87	25.07	40.57	470.18	2.75
Anti-Vi IgG (U/ml)								
All observations	All data	937	469	7.53	27.84	59.01	600.00	7.40
	Cohort 1: Children	317	218	3.02	10.34	23.15	204.60	7.42
	Cohort 2: Adults	620	251	11.32	36.71	69.00	600.00	7.40
Uncensored observations[2]	All data	468	0	24.49	52.78	75.71	600.00	7.40
	Cohort 1: Children	99	0	15.12	28.47	35.20	204.57	7.42
	Cohort 2: Adults	369	0	29.92	59.29	82.11	600.00	7.40

N = total number per group; N cens = numer of observations below the calculation limit (censored values); sd = standard deviation

[1]Two participants had anti-CdtB IgG titres that were repeatedly below the calculation limit; these were excluded from the analysis

[2]robust regression on order statistics were used to calculate summary statistics, due to the high number of

The distribution of antibody responses to the various antigens in serum samples of the randomly selected adults and children is shown in **Figure 7**. These data demonstrated a clear delineation between the distribution of antibody titres between children and adults. For example, the median anti-HlyE IgG titre in Cohort 1 (children) was 351.7 EU, the corresponding median anti-HlyE IgG titre in Cohort 2 (adults) was 198.1 EU, this disparity in antibody titres between children and adults was significantly different (p<0.0001, Wilcoxon test). Similarly, the median anti-CdtB IgG was also significantly higher in children than adults (52.6 vs 12.9 EU; p<0.0001, Wilcoxon test). We also observed a significant difference between the anti-Vi IgG titres in children and adults; however, contrary to the protein antigens, the median anti-Vi IgG titre was higher in adults than children (11.3 vs 3.0 U/ml; p<0.0001, Wilcoxon test).

The anti-HlyE IgG and anti-CdtB IgG titres demonstrated a significant positive correlation with each other (Spearman´s rho = 0.5; p<0.00001) (**Figure 8**). Notably, the correlation coefficient of anti-HlyE IgG and anti-CdtB IgG was substantially higher among children (Spearman´s rho = 0.72; p<0.00001) than among adults (Spearman´s rho = 0.35; p<0.00001). Conversely, the anti-Vi IgG titres did not exhibit a strong correlation with the anti-CdtB IgG titres (all data: Kendall´s tau = -0.09, p=0.14; children: Kendall´s tau = -0.11, p<0.0001; adults: Kendall´s tau = 0.03, p=0.21) or with anti-HlyE IgG titres (all data: Kendall´s tau = -0.03, p=0.14; children: Kendall´s tau = -0.09, p=0.003; adults: Kendall´s tau = 0.03, p=0.004) (**Figure 8**).

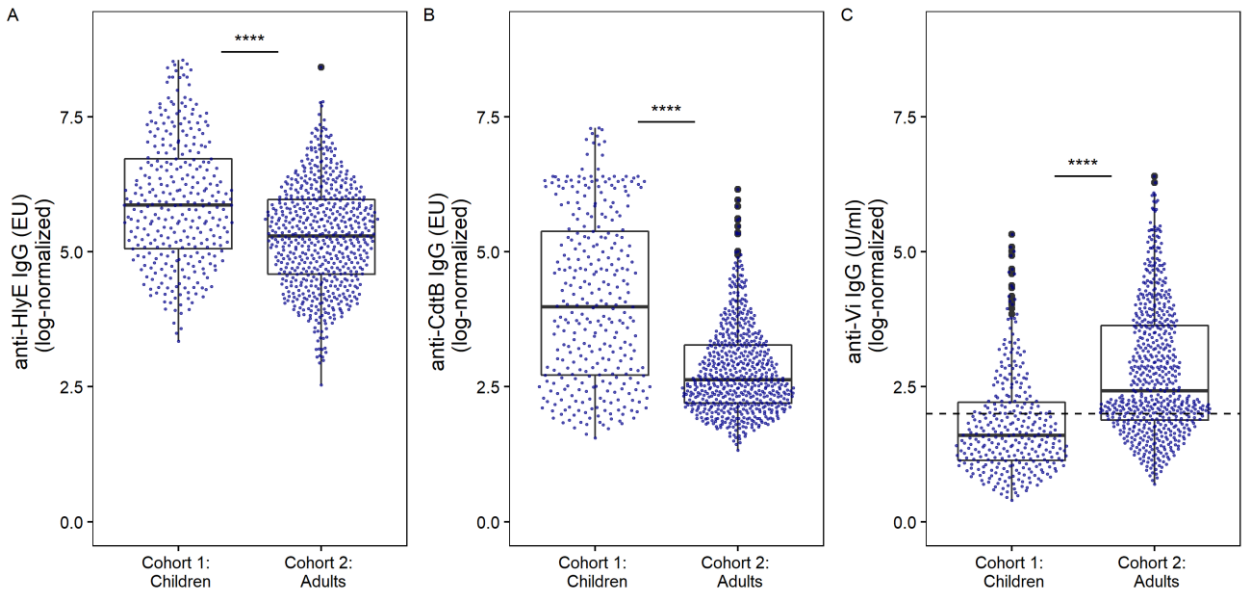

Figure 7 The distribution of anti–S. Typhi serum IgG titers in children and adults in the Lao PDR. Each dot shows the antibody titer of an individual sample for (A) anti-HlyE IgG, (B) anti-CdtB IgG, and (C) anti-Vi IgG with an underlying boxplot. The dashed line in panel C represents the censoring limit, all data points below were treated as left-censored data. Differences between groups were assessed using Wilcoxon rank sum test followed by Dunn's *post-hoc* test with Bonferroni correction: ****p<0.0001.

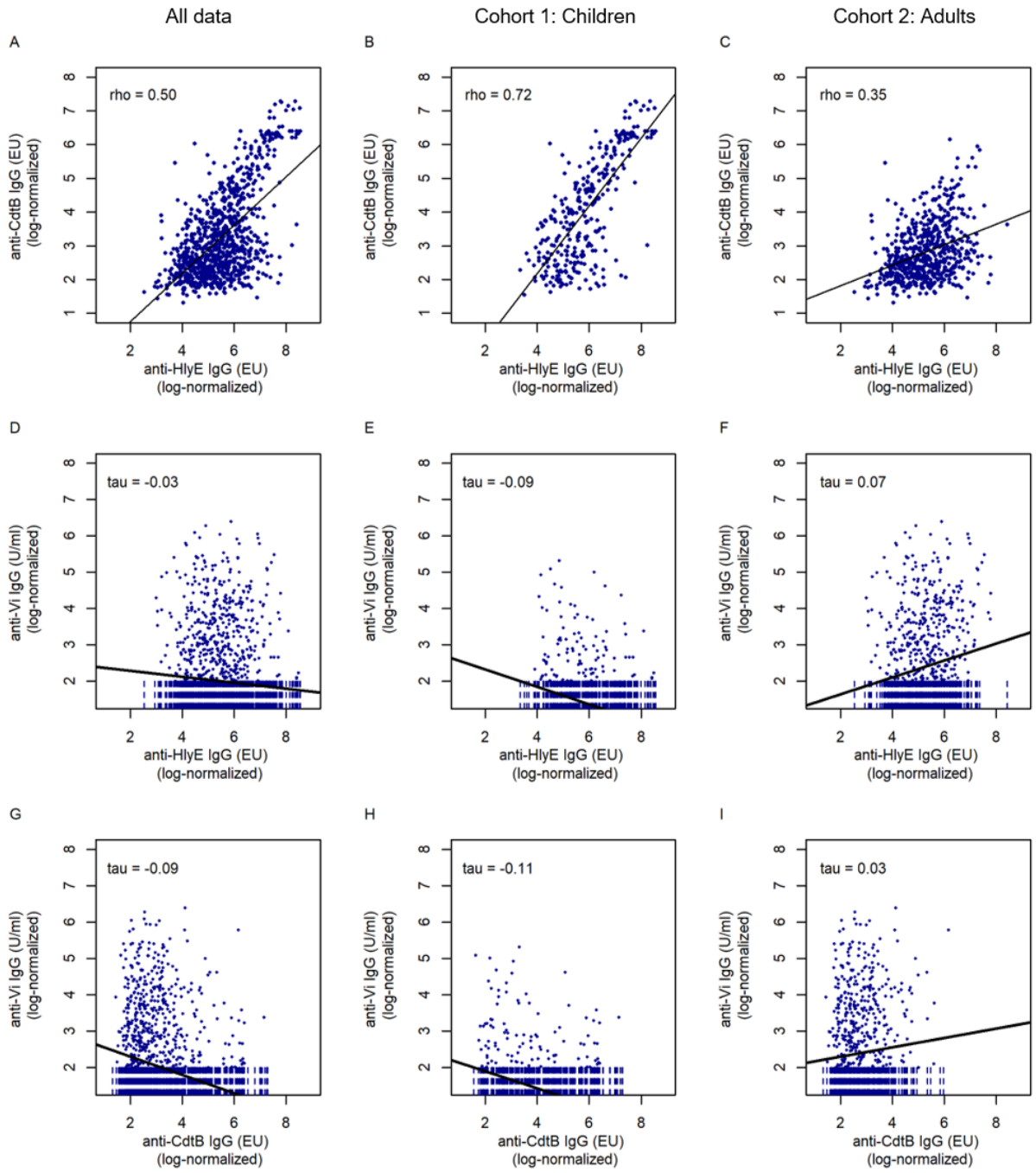

Figure 8 Correlation of anti–S. Typhi serum IgG titers against three antigens in children and adults in the Lao PDR. A, B and C show the correlation between anti-HlyE IgG and anti-CdtB IgG for all data, cohort 1 and cohort 2 respectively. D, E and F show the correlation between anti-Vi IgG and anti-HlyE IgG for all data, cohort 1 and cohort 2 respectively. G, H and I show the correlation between anti-Vi IgG and anti-CdtB IgG for all data, cohort 1 and cohort 2 respectively. Black lines indicate predicted values from linear regression analysis in A, B and C and from Akritas-Thiel-Sen regression lines in D, E, F, G, H and I. Censored observations in D, E, F, G, H and I were plotted as vertical dashed lines.

The relationship between age and anti–S. Typhi serum IgG antibodies

We fitted a linear model to investigate the relationship between anti-HlyE IgG titres and age. We found a significant negative relationship between anti-HlyE IgG titres and age (p<0.0001, adjusted R^2 = 3.4%). To assess the potential of a non-linear relationship between age and anti-HlyE IgG titres, we fitted a generalized additive model (GAM) (**Figure 9**, panel A). The fitted, non-linear trend in the GAM differed significantly (p<0.0001) from the linear trend fitted in the linear regression model (GAM: p<0.0001, adjusted R^2 = 15.5%, deviance explained = 16.2%). When comparing the overall model fit, the GAM demonstrated a better fit with the data than the linear model (GAM: Akaike information criterion (AIC) = 2649.85, Bayesian information criterion (BIC) = 2698.65; linear model: AIC = 2767.8, BIC = 2782.3). Similarly, the GAM describing the relationship between anti-CdtB IgG titers and age (**Figure 9**, panel B) was superior in terms of fit in comparison to the linear model (GAM: p<0.0001, adjusted R^2 = 30.4%, deviance explained = 30.9%, AIC = 2788.01, BIC = 2835.6; linear model: p< 0.0001, adjusted R^2 = 17.4%, AIC = 2939.80, BIC = 2954.32). The GAM for the anti-HlyE IgG and anti-CdtB IgG titres suggested the highest prevalence of antibody was in children aged <5 years (**Figure 9**, panel A and B, **Supplementary Figure 2** in Appendix **C.a**). The fitted GAM trends identified a prominent decrease in antibody titers until the age of 20 years. **Supplementary Figure 3** and **Supplementary Figure 4** (in Appendix **C.a**) show the GAM for anti-HlyE IgG and anti-CdtB IgG antibody titres as a function of age, by province in adults. Despite a visible upward trend of antibody titres with age in some provinces, the trends differ by province.

Lastly, the anti-Vi IgG data was fitted as a function of age, which showed a positive relationship using Akritas–Theil–Sen non-parametric regression to account for the censored data (likelihood r = 0.33, p<0.0001, Kendall`s tau = 0.21; p<0.0001) (**Figure 9**, panel C); suggesting that anti-Vi IgG increases with age.

Trends in anti–S. Typhi serum IgG antibodies with sex, occupation, and study site

Any potential differences in anti-S. Typhi serum IgG antibody titres between female and male participants were assessed using Wilcoxon rank sum test. There was no significant difference for any of the anti-S. Typhi antibodies between male and female children or adults. We next investigated differences in anti-S. Typhi serum IgG antibodies according to occupation and study site in adults. A Kruskal Wallis test revealed a significant difference of anti-HlyE IgG among occupation groups (p<0.0001) (**Supplementary Figure 5** in Appendix **C.a**). A *post-hoc* Dunn´s test with Bonferroni correction determined significant differences between the general population with unspecified occupation (“other”) (median anti-HlyE IgG = 312.4 EU) and students (median anti-HlyE IgG = 157.6 EU), between office workers (median anti-HlyE IgG = 259.2 EU) and students, and between office workers and soldiers (median anti-HlyE IgG = 187.5 EU). Likewise, there was a significant difference between anti-CdtB IgG titres in students (median anti-HlyE IgG = 10.1 EU) and soldiers (median anti-

HlyE IgG = 13.2 EU), the general population with unspecified occupation (median anti-HlyE IgG = 17.0 EU)) and students and between students and office workers (median anti-HlyE IgG = 15.2 EU) There was no significant difference in anti-Vi IgG titres between the occupation groups.

Further Kruskal Wallis tests revealed significant differences in anti-HlyE IgG titres, anti-CdtB IgG titres, and anti-Vi IgG titres between the different provinces ($p<0.0001$, $p<0.01$ and $p<0.05$ respectively) (**Figure 10**). A *post-hoc* Dunn´s test with Bonferroni correction identified a significant difference between several provinces. The distribution of anti-HlyE IgG titres differed between Khammuane (median anti-HlyE IgG = 255.9 EU) and Phongsaly (median anti-HlyE IgG = 144.0 EU), Attapeu (median anti-HlyE IgG = 142.3 EU) and Xayabouli (median anti-HlyE IgG = 157.8 EU), and between Vientiane (median anti-HlyE IgG titer = 272.0 EU) and Attapeu and Xayabouli (**Figure 10**, panel A). The median anti-CdtB IgG titres differed significantly between Khammuane (median anti-CdtB IgG = 15.3 EU) and Xayabouli (median anti-CdtB IgG = 9 EU) and between Vientiane (median anti-CdtB IgG = 16.1 EU) and Xayabouli (**Figure 10**, panel B).

The only significant difference in median anti-Vi IgG titres was identified between Khammuane (median anti-Vi IgG = 20.3 U/ml) and Luang Prabang (median anti-Vi IgG = 7.8 U/ml) (**Figure 10**, panel C)

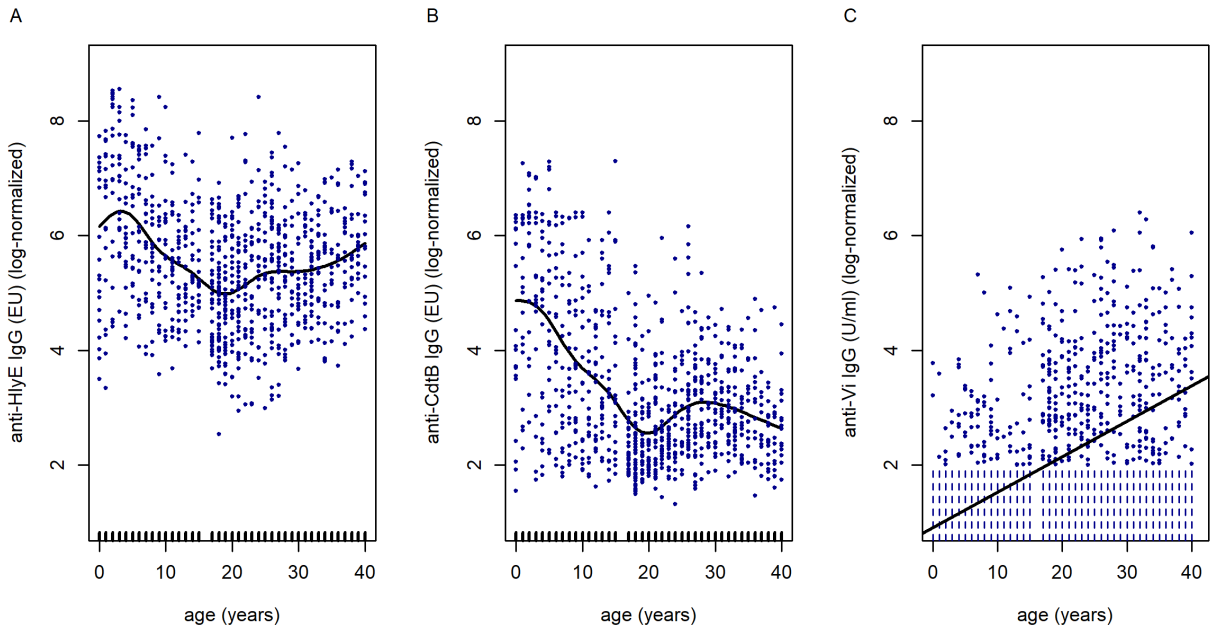

Figure 9 Results of generalized additive and linear models assessing anti–S. Typhi IgG antibody prevalence in children and adults in the Lao PDR as a function of age. Non-linear smooths were fitted for age in the model for anti-HlyE IgG (A) and anti-CdtB IgG (B) data. The tick marks on the x-axis are observed data points. In panel C, the Akritas-Thiel-Sen regression line relating to the anti-Vi IgG titer data as function of age was plotted in order to account for the censored values (censored observations were plotted as vertical dashed lines).

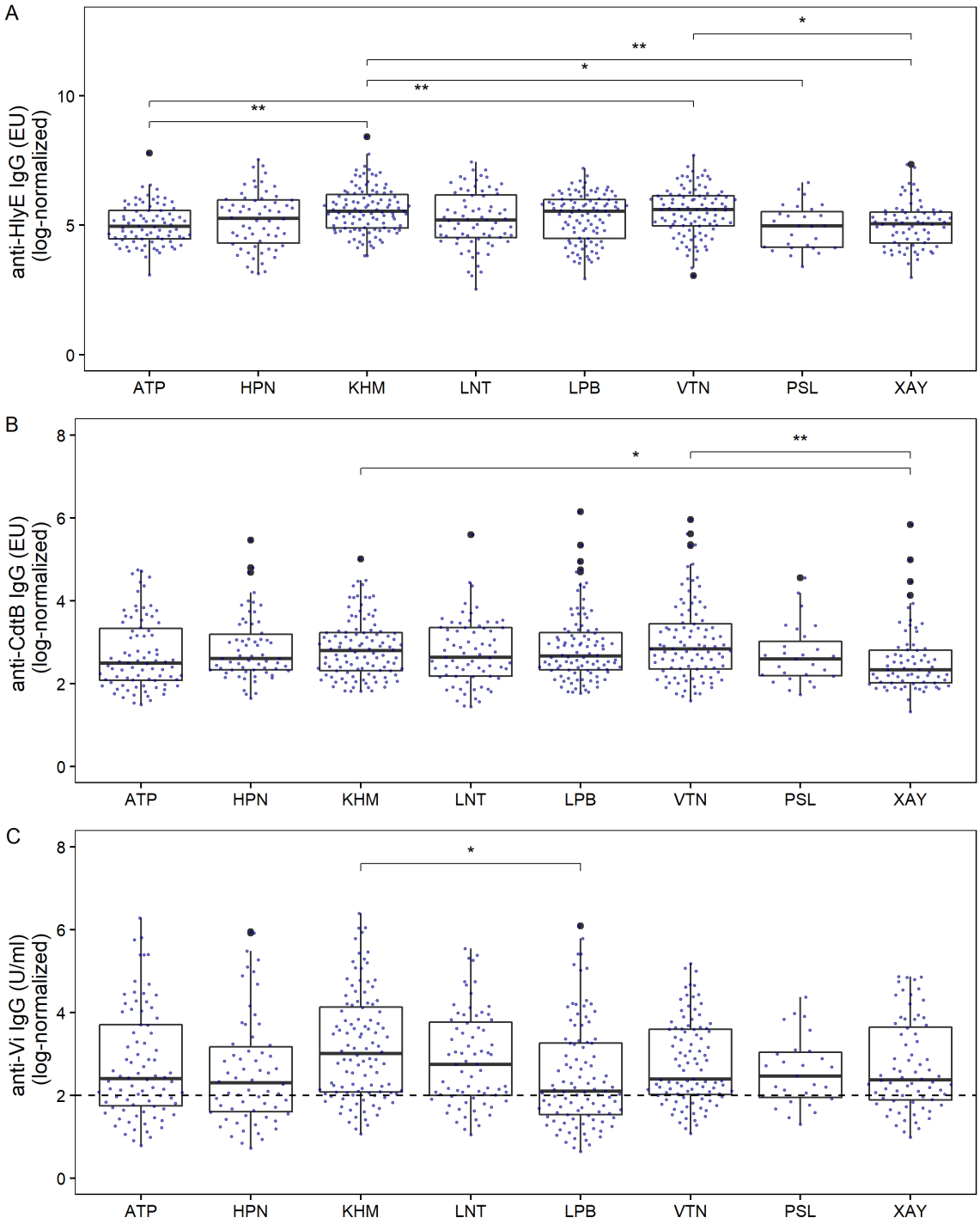

Figure 10 The distribution of anti–*S.* Typhi serum IgG titers in adults in the Lao PDR by province. Each dot shows the measurement of an individual sample for (A) anti-HlyE IgG, (B) anti-CdtB IgG and (C) anti-Vi IgG with an underlying boxplot. Differences between groups were assessed using Kruskal-Wallis test followed by Dunn's *post-hoc* test with Bonferroni correction: *p<0.05, **p<0.01. ATP = Attapeu, HPN = Huaphan, KHM = Khammuane, LNT = Luang Namtha, LPB = Luang Prabang, VTN = Vientiane, PSL = Phongsaly, XAY = Xayabouli. The dashed line in panel C represents the censoring limit, all data points below were treated as left-censored data.

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Discussion

This study offered first insights into the age-related prevalence of three antibodies against *S.* Typhi in Lao PDR.

Antibodies against the Vi antigen are most commonly used in serological assays. In this study, 50.1% of the participants had anti-Vi IgG antibody concentrations <7.4 U/ml with an estimated median titre of 7.5 U/ml. Median baseline concentrations were higher in adults as compared to children (11.3 vs 3.0 U/ml) and a positive relationship of anti-Vi IgG titre and age was observed. These findings are consistent with other reports [87,126]. The median anti-Vi titre without those measurements below the calculation limit was 24.5 U/ml in general and 29.9 U/ml among adults, which was higher than previously reported median titres of 8.6 U/ml and 21 U/ml in healthy adults from Spain and Germany [26,100].

In contrast to the anti-Vi IgG data, the median anti-HlyE IgG and anti-CdtB IgG titres were higher in children than in adults. The non-linear trend fitted to the HlyE IgG data suggested a peak in concentration in children below 5 years of age, followed by a decrease and subsequent increase again after the age of 20 years. The anti-CdtB IgG data followed largely the same projection; however, an increasing trend after the age of 20 years was not observed. Both anti-HlyE IgG and anti-CdtB IgG correlated reasonably well with each other, both in the adult and children cohort. The anti-Vi IgG data, however, did not correlate well with either of the other two antibody profiles.

The prevalence of antibodies developed against the two protein antigens, anti-HlyE IgG and anti-CdtB IgG, indicates a high exposure in children. These findings are somewhat in contrast to a hospital-based surveillance study in Vientiane which reported no incidents of typhoid fever in children over the course of two years [12]. In a retrospective study in a CH in Vientiane, spanning the time from 2000 to 2018, the median age of patients with confirmed typhoid fever was 21 years [98].

Students had lower medium anti-CdtB and anti-HlyE IgG titres than other occupation groups in the adult cohort, which likely reflects our findings that the antibody response against the protein antigens decreased until the age of 20 years. The highest medium anti-CdtB and anti-HlyE IgG titres were reported from Vientiane and Khammune province among the adult participants. Likewise, the highest medium anti-Vi IgG titre was found in Khammuane.

Our study revealed a high anti-*S.* Typhi prevalence in children and the findings in the adult cohort indicate regional variability. Based on our study, we suggest the introduction of Typhoid conjugate vaccines (TCV) in children. Nevertheless, more data is needed to fully characterize the typhoid fever disease burden in the Lao PDR. A follow-up study including children from several provinces and age groups could provide more information on typhoid fever disease burden in different areas of the Lao PDR.

This study has several limitations. First of all, the samples selected for this study were collected in different years in the framework of other studies. The data does not include information whether the participants had ever been diagnosed with typhoid fever before or any other health-related information. The sample collections in these previous studies were not randomized and therefore may limit our ability to generalize the findings. Serum samples from children was only available from two provinces in the Lao PDR. Furthermore, samples were collected at one time point only and represent a cross-sectional view.

Acknowledgments

We wish to acknowledge the investigators, especially Vilaysone Khounvisith and Dr Phonethipsavanh Nouanthong, from the named studies for providing access to the samples and the study participants. We are thankful to our collaborators at Luxembourg Development Cooperation, the Lao Red Cross and Provincial and district hospitals and health centres. We are grateful to Latdavone Khenka and Bounta Vongphachanh, who helped with the experiments. We wish to acknowledge Dr Paul Brey and staff at the Institut Pasteur du Laos for their support.

CHAPTER 3. *Haemophilus influenzae* serotype b seroprevalence in Central provinces in the Lao PDR before and after vaccine introduction

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3.1. Contribution statement

The study was conceived by Dr Black and Ms Hefele. Sample testing and data analyses were conducted by Ms Hefele. The initial draft of the manuscript was written by Ms Hefele. All authors wrote and commented on the manuscript. The supplement material can be found in Appendix **C.b**.

The results presented in this study will be combined with research work on *H. influenza*e type b in Lao PDR by Dr Jana Lai, but for the purpose of this thesis, only the results by this research group are shown. The research manuscript combining both the data presented here and additional data is currently under preparation for submission to a peer-reviewed journal.

3.2. Bridging section

At present, there is no study reporting clinical data on Hib in the Lao PDR and there is also no research article regarding Hib seroprevalence other than the one presented in this thesis in another chapter (**CHAPTER 4**). Hib vaccination was introduced in the Lao PDR in 2009 as part of the pentavalent vaccine containing purified capsular Hib polysaccharide (PRP) conjugated to the tetanus toxoid (carrier protein). In the study presented in this chapter, the seroprevalence of anti-Hib antibodies was investigated before and after the introduction of the vaccination.

The research in this chapter contributes to two research objectives as stated in section **1.8.1**, namely to characterize of the epidemiology of VPDs for which there is (or in this case: *was*) no routine vaccination in the Lao PDR and to assess the level of seroprotection against VPDs and to investigate possible risk factors or predictors for seroprotection.

The prevalence of anti-Hib antibodies was investigated in a sample of Lao adolescents, born before 2009, in order to understand disease susceptibility in the pre-Hib-vaccination era. These data may serve as baseline data to which post-vaccination data can be compared. In addition, serum samples from a cohort of vaccinated children and a cohort of children with mixed vaccination status was screened for Hib-antibodies to estimate vaccine-derived immunity against Hib and to identify possible predictors for long-term protection.

3.3. Abstract

Introduction

Vaccination has dramatically reduced invasive *Haemophilus influenzae* type b (Hib) disease worldwide. Hib vaccination was introduced in the Lao PDR in 2009, as part of the pentavalent vaccine. To contribute to the understanding of the epidemiology of Hib in the Lao PDR and the protection levels before and after the introduction of the vaccination, we tested serum samples from existing cohorts of children and adolescents for antibodies against Hib.

Methods

Serum samples from 196 adolescents (11-18 years) born before vaccine introduction and from 761 children under 5 years (vaccinated and unvaccinated) were tested for anti-Hib antibodies by ELISA. Antibody titers between 0.15-1 µg/ml were considered as evidence for exposure to the pathogen and/or short-term protection and titers >1 µg/ml were classified as long-term protection. Bivariate analyses were performed to investigate factors associated with long-term protection.

Results

The vast majority of all participants showed evidence of short- (43.3%) or long-term (55.2%) protection against Hib. Almost all of the unvaccinated adolescents had antibody titers indicating short-term protection (95.9%) and almost half (45.6%) were long-term protected. Nearly all of the children (>99.0%) were at least short-term protected. Among vaccinated children, participants vaccinated more than 1 or 2 years before the sample collection were less likely to be long-term protected.

Discussion

Nearly all of the adolescents born before the introduction of Hib vaccination in the Lao PDR had antibody titers corresponding to short-term protection; indicating a high burden of Hib disease at that time. After vaccine introduction, all vaccinated children showed at least short-term protection; but only 58.9% were long-term protected, indicating low vaccine immunogenicity and/or rapid antibody waning. Our results highlight the need for robust surveillance and reporting of invasive Hib disease to determine the current burden of disease.

3.4. Manuscript

Introduction

Haemophilus influenzae type b (Hib) causes pneumonia and meningitis almost exclusively in children under 5 years of age [127]. Before widespread vaccination in 2000, Hib was responsible for at least 8.13 million cases of serious disease in children (<5 years) and 371 000 deaths globally. Vaccination has dramatically reduced invasive Hib disease worldwide [147]. The pentavalent vaccine DTPw-HepB-Hib (Diphtheria-Tetanus-Pertussis (whole cell)-Hepatitis B-*Haemophilus influenzae* type b), containing purified capsular Hib polysaccharide (PRP) conjugated to the tetanus toxoid (carrier protein), was introduced in the Lao PDR in 2009, replacing the DTPw-HepB vaccine. DTPw-HepB-Hib is scheduled at 6, 10 and 14 weeks of age [91]. In 2018, the coverage with the DTPw-HepB-Hib vaccine was 84% in the Lao PDR[157]. Immunity against Hib can be determined by measuring antibody levels against the PRP immunogenic component of Hib conjugate vaccines [93].

There are little data on the level of immunity against Hib or the burden of disease in the general population in the Lao PDR. Two serosurveys conducted in Bolikhamxay province found that nearly

66.4% and 71.7% of children 8 to 28 months old had long-term Hib antibody protection (>1µg/ml) in 2013/14 and 2017 respectively[40].

To contribute to the understanding of the epidemiology of Hib in the Lao PDR and the protection levels before and after the introduction of the vaccination, we tested serum samples from existing cohorts of children and adolescents for antibodies against PRP. We also investigated possible predictors for long-term protection.

Methods

Participants

Cohort 1: Unvaccinated adolescents

Serum samples of 296 students from Bolikhamxay province and Vientiane Capital, collected in 2018 in the framework of another study [41] were selected from a total of 779 students (**Figure 11**). All participants in this study were born before 2008, before Hib was introduced into the national immunization programme and therefore were most likely not vaccinated against Hib. Students between the age of 11 and 18 years were selectively randomized in order to have the same age and sex ratios in both provinces. Socio-economic data (i.e. district, age, sex, ethnicity, place of birth and number of household members) were collected using a standardized questionnaire. The study was approved by the Lao National Ethics Committee (Reference number 022/NECHR) and the Institutional Review Board of the Institut Pasteur du Laos (Reference number 9).

Cohort 2: Vaccinated Children

This cohort consists of 761 children from Vientiane, Bolikhamxay and Khammuane province aged 9 to 50 months. All children had records of three doses of the pentavalent vaccine, as confirmed by reviewing the vaccination log books at the health care facilities and were recruited in the context of another study in 2013/14 [28] (**Figure 11**).

Even though the anti-Hib data of children from Bolikhamxay between 8 and 28 months (n=140) were reported in a previous study [40]; the entire cohort (n=228) were not reported before nor in conjunction with the other cohorts presented here. Socio-economic and health related data (i.e. district, age, sex, place of birth and date of vaccination etc) were collected using a standardized questionnaire. In addition, the nutritional status of the children was determined by mid-upper arm circumference, weight for height, height for age, weight for age and body mass index z scores as described in the previous publication[28]. The study was approved by the Lao National Ethics Committee (NECHR2013-860).

Laboratory analyses

Serum samples were tested at the IPL for the presence of anti-PRP IgG using the commercial ELISA kit IMMUNOZYM (Progen) according to the manufacturer´s protocol. Antibody concentrations were derived from the optical density (OD) data using a standardized curve-fitting 4-parameter logistic method. Any sample above the calculation limit of the assay was given the value of 5.35 µg/ml, which corresponds to the concentration of the highest calibrator in this assay, for the purpose of analysis. Antibody titres below 0.15 µg/ml were considered as insufficient protection, titres between 0.15 and 1 µg/ml were considered as evidence for exposure to the pathogen and short-term protection and titres above 1 µg/ml were classified as sufficient immunity (long-term protection).

Figure 11 Map of study sites in the Lao PDR. Districts in which samples were collected are highlighted. The map was created with QGIS (QGIS Development Team, 2018) using collected GPS-data and the world borders dataset (http://thematicmapping.org/downloads/world_borders.php, 2019). The data regarding the administrative boundaries of the Lao PDR was obtained from the Humanitarian Data Exchange website (https://data.humdata.org/dataset/lao-admin-boundaries, dataset provided by the National Geographic Department of Lao PDR, 2019). Projection used: EPSG 4326 – WGS 84.

Data analyses

Data analyses were conducted using R statistical software [94] with the following packages: tidyverse [131], MASS [123], car [31], haven [129], lubridate [35], stringr [130] and epitools [7]. In bivariate analyses, odds ratio, 95% confidence intervals (CI) and p values were calculated to investigate factors associated with long-term protection. Chi-squared and Fisher´s exact tests were used as appropriate. Shapiro-Wilks goodness-of-fit test was used to assess the normality of data and the correlation between two numerical variables was assessed by calculating the Spearman rank correlation coefficient rho. A p value <0.05 was considered statistically significant.

Results

Participants' characteristics

In total, 1057 participants from the two cohorts were included in this study (**Table 4**). The majority of the participants in cohort 1 were of Tai-Kadai ethnicity, which is the main ethnic group in the Lao PDR. Information on ethnicity was not available for cohort 2. Serum samples of all two cohorts were collected in provinces located in central Lao PDR (**Table 4**). Since all adolescents (cohort 1) were born before 2008, it was assumed they would not have received routine infant Hib vaccination. All children included in cohort 2 had written records of a full course of the DTPw-HepB-Hib vaccine.

Prevalence of anti-Hib IgG

The vast majority (>90%) of the participants showed evidence of short- (43.3%) or long-term (55.2%) protection against Hib (Appendix **C.b**, **Supplement Table 1**).

Almost half (45.6%) of the unvaccinated adolescents (cohort 1) had an anti-Hib IgG titer >1.0 µg/ml corresponding to long-term protection. Nearly 60% of cohort 2, all born after the introduction of the pentavalent vaccine, showed long-term protection (Appendix **C.b**, **Supplement Table 1**).

Factors associated with long-term protection

Even though the proportion of participants with long-term protection varied between age groups at the different locations, there was no statistical difference between the respective youngest and older age groups (**Figure 12**).

In the cohort of unvaccinated adolescents (cohort 1), being long-term protected (anti-Hib IgG >1 µg/ml) was not associated with study location (district or province), sex, age, place of birth, number of household members or ethnicity.

In vaccinated children (cohort 2), time since vaccination was associated significantly with being long-term protected. Participants vaccinated more than 1 or 2 years before the sample collection were less likely to have anti-Hib IgG >1.0 µg/ml (55.6% vs 66.1%; OR=0.6; p=0.01 and 53.4% vs 66.1%;

OR=0.6; p=0.02 respectively). Age and time since vaccination were also significantly correlated with antibody titres (rho = -0.08, p<0.05 and rho =-0.11, p<0.01, respectively). Furthermore, participants with a mid-upper arm circumference z-score <-2 were slightly less likely to be long-term protected (40.5% vs 59.8%; OR=0.5; p=0.03). None of the other factors were positively or negatively associated with long-term protection.

Table 4 Characteristics of study participants by cohorts.

		Unvaccinated Adolescents (Cohort 1)	Vaccinated children (Cohort 2)
Number of participants		296	761
Sample collection		2018	2013 -2014
Age (years)	Median (IQR*)	15 (13-17)	1.8 (1.2-2.3)
	Mean	14.6	1.9
Study Location n(%)	Vientiane Capital	148 (50.0)	0 (0)
	Vientiane Province	0 (0)	178 (23.4)
	Bolikhamxay Province	148 (50.0)	228 (30.0)
	Khammuane Province	0 (0)	355 (46.7)
Sex n(%)	Male	141 (47.6)	381 (50.1)
	Female	155 (52.4)	380 (49.9)
Ethnicity n(%)	Tai Kadai	283 (95.6)	na
	Hmong-Mien & Mon-Khmer	13 (4.4)	na
Hib vaccination status n(%)	Full course (documented)	na	761 (100)
	Incomplete course	na	0 (0)
	No vaccination	na	0 (0)
	Unknown (no documentation)	296 (100)	0 (0)
*IQR = Interquartile range			

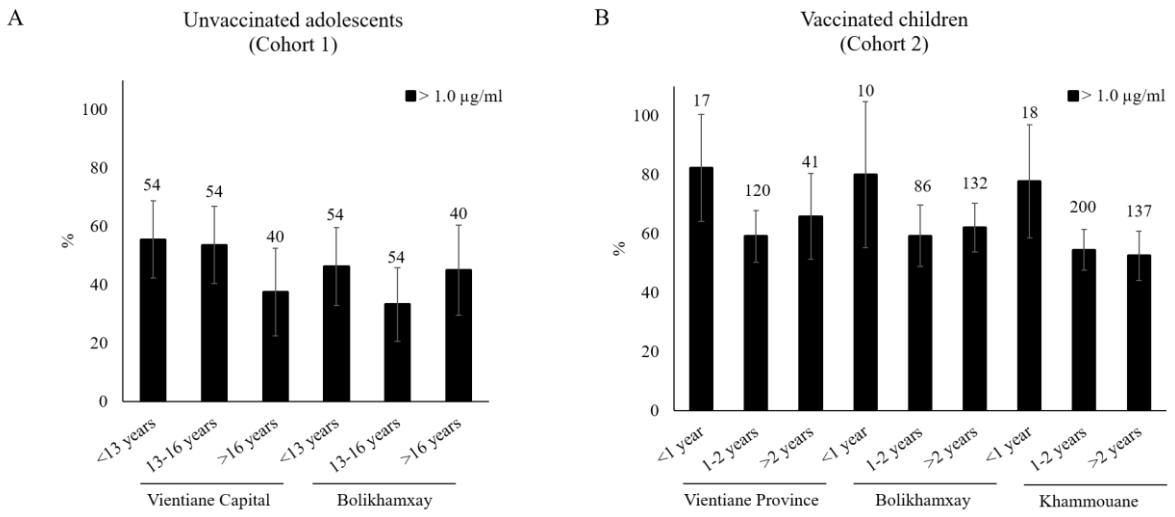

Figure 12 Proportion of participants with long-term protection (anti-Hib IgG >1 µg/ml) according to age group and study location. A) Unvaccinated adolescents (cohort 1). B) Vaccinated children (cohort 2). The number above the bars represent the total number per group.

Discussion

To the best of our knowledge there is no information about the anti-Hib IgG protection levels or the incidence of pneumonia or meningitis due to Hib prior to the introduction of the vaccine in the Lao PDR. In this study, we found high long-term protection rates in adolescents born before the introduction of the vaccine. Virtually all unvaccinated adolescents in cohort 1 showed evidence of natural exposure to *H. influenzae* type b suggesting that there may have been a substantial burden of disease before vaccine introduction in 2009. Our findings are similar to other serosurveys assessing antibody levels before the introduction of the vaccination. A study conducted in Nepal in the pre-Hib-vaccination era reported a prevalence of >0.15 µg/ml for at least 67% of the participants in Kathmandu before adulthood. In that study, 20% of children under five years of age had anti-Hib IgG >0.15 µg/ml which increased to 83% among 15-54 year olds [69].

After the introduction of Hib vaccination, virtually all children in cohort 2 showed short- or long-term protection. All children in cohort 2 received all three doses of DTPw-HepB-Hib vaccine and should therefore be long-term protected; however, 41.1% in cohort 2 had antibody titres insufficient for long-term protection. We reported low levels of antibodies against diphtheria, tetanus and hepatitis B without a clear explanation in cohort 2 before [28]. The follow-up study that we conducted in 2017 revealed that vaccine immunogenicity was especially low in remote areas, indicating problems in vaccine management, but also showed a significant improvement in seroprotection rates compared to the study in 2013/14 in children aged 8 to 28 months The proportion of children protected against Hib rose about 5% from 2013/14 to the study in 2017, however, the difference was not significant [40].

Another reason for the large proportion of short-term protected children in cohort 2 could be rapid waning of anti-Hib antibodies. Indeed, time since vaccination was associated with having a lower antibody titre in cohort 2 in this study. Hib antibody waning has been reported previously and the introduction of a booster dose into the vaccination schedule has been discussed without a conclusion due to lack of data [38,106,147]. Furthermore, since data quality in the health sector has been of concern in Lao PDR before, we cannot exclude the possibility that the participants had not received the vaccination despite having documentation [112].

There were no significant differences in long-term protection rates by location, ethnicity or most of the other factors within the different cohorts, except for time since vaccination and one of the nutritional parameters. A weak association between seroprotection against diphtheria and malnutrition was reported in cohort 2 before [28]. Generally, it seems as if vaccination can induce adequate immune responses (albeit possibly reduced) in malnourished children, also in the context of a conjugated Hib vaccine. However, the interplay between immunity and malnutrition is not clearly characterized yet. It is also not clear, if malnutrition impacts the duration of immunity or the generation of an immune memory [92].

Antibody waning and continued exposure to *H. influenzae* type b could lead to increasing infection rates. More data are needed to evaluate whether the vaccination led to a reduction in Hib carriage in children. However, other countries have reported a decrease of confirmed Hib related diseases after vaccine introduction [37,70,110]. Clinical data in addition to the monitoring of vaccine-induced protection rates could provide helpful information whether a booster is needed, using this study as baseline.

Limitations of our study may result from the different study designs of the original studies and laboratory methods. Participants in cohorts 2 were not selected randomly. Our findings are from Central areas of the Lao PDR. Hib prevalence may vary between regions of the Lao PDR and/or between different ethnic groups.

Conclusion

Our findings indicate that the circulation of Hib was high in the Lao PDR before the introduction of the vaccine. Indeed, after vaccine introduction, all vaccinated children showed serological markers of vaccination/past infection and protection. Robust surveillance and reporting of invasive Hib diseases is needed to determine the current burden of disease; several years after vaccine introduction.

CHAPTER 4. Seroprotection on different levels of the health care system after vaccination with DTPw-HepB-Hib in the Lao PDR

4.1. Contribution statement

The study presented in this section was published as:

Lisa Hefele, Sengdavanh Syphan, Dalouny Xayavong, Anousin Homsana, Daria Kleine, Phetsavanh Chanthavilay, Phonethipsavanh Nouanthong, Kinnaly Xaydalasouk, Outavong Phathammavong, Somxay Billamay, Anonh Xeuatvongsa, Daniel Reinharz, Claude P Muller, Antony P Black, *Seroprotection at Different Levels of the Healthcare System After Routine Vaccination With Diphtheria-Tetanus-Pertussis whole cell–Hepatitis B–Haemophilus influenzae Type B in Lao People's Democratic Republic*, Clinical Infectious Diseases, Volume 69, Issue 12, 15 December 2019, Pages 2136–2144, https://doi.org/10.1093/cid/ciz143

Ms Hefele supervised sample collection, data acquisition and laboratory testing of the samples collected in Bolikhamxay province; conducted data cleaning, primary and final data analysis, summarized the findings, wrote the initial draft and wrote and edited on the manuscript. An author contribution statement signed by all authors was supplied separately. The supplement material can be found in Appendix **C.c**.

4.2. Bridging section

In the previous study (**CHAPTER 3**), serology was used to gain insight into the epidemiology of Hib in absence of routine vaccination and identify potential risk factors or predictors of long-term protection. In the study described in this chapter, serology data is used to monitor vaccine-induced immunity in order to contribute to the evaluation of immunization programme performance. Typically, vaccination coverage rates are used to measure the performance of immunization programmes; however they do not inform on population immunity conferred through vaccination and depending on the reporting system, they may not include information regarding the timeliness of vaccination. Serological data can provide information regarding age-specific population immunity and in some cases, it is possible to differentiate directly between natural and vaccine-induced immunity. Serological investigations after vaccine introduction can help estimate the duration of immunity, detect immunity gaps, reveal problems with vaccine immunogenicity and identify vulnerable populations. In the study presented in this chapter, the immunogenicity of the pentavalent DTP-HepB-Hib in vaccinated children was assessed and risk factors and predictors for seroprotection and seropositivity were investigated.

4.3. Publication

Clinical Infectious Diseases

MAJOR ARTICLE

Seroprotection at Different Levels of the Healthcare System After Routine Vaccination With Diphtheria-Tetanus-Pertussis whole cell–Hepatitis B–*Haemophilus influenzae* Type B in Lao People's Democratic Republic

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Background. The Lao People's Democratic Republic continues to sustain a considerable burden of vaccine-preventable diseases because of incomplete vaccine coverage and weak vaccine responses. We have assessed seroconversion after routine vaccination with the pentavalent vaccine to capture weaknesses of vaccine management at the different levels of the healthcare system.

Methods. A total of 1151 children (aged 8–28 months) with 3 documented doses of the pentavalent vaccine delivered at central hospitals in Vientiane and the provincial hospital, 3 district hospitals, and 10 health centers in Bolikhamxay province were enrolled. Sociodemographic information was collected with a standardized questionnaire. Serum samples were analyzed for antibodies against vaccine components, and bivariate and multivariable analyses were performed to identify risk factors for low vaccine responses.

Results. Seroprotection rates at the provincial, district, and health center level were as high as in central hospitals, but seroprotection rates in areas covered by remote health centers were significantly lower. Protective levels also rapidly decreased with age at sampling. Seroprotection rates in Bolikhamxay against the different components reached 70%–77% and were up to 20% higher than in previous studies in the same region; 18.8% more children received the hepatitis B vaccine birth dose and the hepatitis B virus infection rate was 4 times lower.

Conclusions. Vaccine immunogenicity has dramatically improved in a central province, likely due to training and investment in the cold chain. Nevertheless, there remains a need to focus on the "last mile" in remote areas were most children are vaccinated through outreach activities.

Keywords. immunogenicity; vaccination; hepatitis B; diphtheria; tetanus.

Despite improvements in healthcare in the Lao People's Democratic Republic (PDR), the incidence of vaccine-preventable diseases remains high. Vaccination represents one of the most important public health interventions [1–3], but many children fail to respond to the pentavalent diphtheria-tetanus-pertussis (whole cell)–Hepatitis B–*Haemophilus influenzae* type B vaccine (DTPw-HepB-Hib) vaccine [4], which is scheduled at 6, 10, and 14 weeks after birth. A first dose against hepatitis B is given as a monovalent vaccine within 24 hours after birth ("birth dose"). Long-term protective titers against hepatitis B virus (HBV; ≥100 IU/L) were as low as 7.7% in children aged 8–28 months with 3 documented vaccinations in 2013/2014 [4]. Many reasons can account for vaccine failure. However, in the 2013/2014 study, the low levels of seroprotection could only be partially explained by rapid waning of antibodies, home birth, malnutrition, and potential geographic disparities. The sensitivity of vaccines to excessive heat or cold is a major challenge for immunization programs. Exposure of vaccines to freezing temperatures resulted in vaccines with impaired immunogenicity [5–8].

Interventions from the Ministry of Health (MoH), World Health Organization (WHO), UNICEF, the Luxembourg Development Agency (LuxDev), and others have prompted a thorough review of vaccine management within the healthcare system. In Lao PDR, healthcare is delivered at 4 levels. The central hospitals (CHs) in Vientiane represent the first level,

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followed by the provincial health offices, each supervising a provincial hospital (PH), and a number of district health offices and district hospitals (DHs). The health centers (HCs) that belong to the DHs represent the lowest level, providing basic medical and outreach services to the surrounding villages [9].

The distribution of vaccines follows the same administrative line. Upon arrival in the country, the vaccines are stored in a central storage facility in Vientiane, from where the facilities of the capital and the provincial levels are supplied. The provincial health office supervises distribution to the DHs from where connected HCs are supplied. Immunization services are offered either directly at the facilities or through outreach services [10, 11].

Considering previous weaknesses of the immunization program and recent interventions, we reassessed the immunogenicity of the pentavalent vaccine in Bolikhamxay. We included children with 3 documented vaccine doses. A particular focus of our study was the site/level within the healthcare system at which the vaccine was delivered in order to capture any systemic logistical issues. We also included children vaccinated at CHs in Vientiane that are a short distance from the central storage facilities, as a surrogate gold standard for other levels of the health system.

METHODS

The study was approved by the Lao National Ethics Committee and by the internal ethics review board of the Institut Pasteur du Laos. The numbers of the ethical approval documents are (033/2017/NECHR, 032/2017/NECHR, 031/2017/NECHR, 056/2017/NECHR).

Study Participants

Children were recruited from the Children's Hospital, a CH in Vientiane and from the PH in Paksan; 3 DHs in Viengthong, Khamkheut, and Pakkading; and 10 HCs in Bolikhamxay (Supplementary Figure A1). All parents/guardians signed the informed consent form and could withdraw participation at any time. A standardized questionnaire was designed to collect information about the participant's socioeconomic background, access to healthcare, history, and location of vaccination. Vaccination histories were verified in hospital records (HRs) at the healthcare facilities (HCFs) if available and/or on the vaccination cards.

At the Children's Hospital, 319 children aged 8–23 months who had received all 3 doses of the pentavalent vaccine in a CH in Vientiane were enrolled between May 2017 and February 2018. Of 319 children, 209 (65.5%) were vaccinated with all 3 doses at the Children's Hospital, 36 (11.3%) received 1 or 2 of their doses at another CH, and 74 (23.2%) received all 3 doses at another CH. We reviewed the vaccination cards for all participants. A digital version of the questionnaire was used to collect the information [12].

In Bolikhamxay Province, 819 children aged 8–28 months vaccinated with the pentavalent vaccine were recruited between March 2017 and July 2017. Each of the 3 injections was documented by the vaccination card (73.3%), the HR (83.2%), or both (56.4%). HCFs and villages were selected based on geographic location, population size, and travel time to the next highest-ranked facility. Participants recruited at a particular HCF were almost always also vaccinated there (93.2%), receiving all 3 doses either on site or through outreach. Nevertheless, some of the participants (6.8%) had been vaccinated for 1 or more of their doses at another HCF or by a mix. All data were double entered in EPIDATA version 3.1 [13].

Serology

After informed consent, 5 mL of blood were collected by trained healthcare workers. Serum was separated by centrifugation on the day of collection and stored at 4°C for a maximum of 5 days and then at −20°C for a maximum of 2 months. Commercial enzyme-linked immunosorbent assay kits were used to determine antibodies against hepatitis B surface antigen (anti-HBs), hepatitis B core antigen (anti-HBc) (Diasorin), tetanus, and diphtheria (Euroimmun). Anti-HBc(+)/anti-HBs(−) sera were also tested for the presence of the hepatitis b surface antigen (HBsAg) (Diasorin). The results were compared to those for the sera from our 2013/2014 study, which were tested with the same kits in the same laboratory at the Institute Pasteur du Laos. In addition, samples collected in the present study were tested for antipertussis toxin (Euroimmun) and anti-Hib (Progen) in parallel with the sera from 2013/2014. Immunity was considered protective when participants had antidiphtheria titers ≥0.1 IU/mL, antitetanus titers >0.5 IU/mL, and anti-Hib titers >1.0 μg/mL. For the anti-HBs titers, 2 cutoffs were used: ≥10 IU/L and ≥100 IU/L (long-term protection). To date, there is no protective titer established for antibodies against *Bordetella pertussis*, which complicates the interpretation of these titers. Here, a titer of ≥22 U/mL was used as an indication of exposure either to the vaccine antigen or the pathogen [14], but the assay cannot differentiate between the two.

Data Analyses

Data analyses were conducted using R software [15] with the following packages: tidyverse [16], pastecs [17], epitools [18], car [19], lmtest [20], MASS [21], and rcompanion [22]. As we were interested in the association between the place of vaccination and seroprotection in semiurban and rural areas, only those participants enrolled in Bolikhamxay who had received all doses of the vaccine at the same place were included in the regression analyses. In bivariate analyses, odds ratio, 95% confidence interval, and *P* value for seroprotected children were calculated. Only variables with *P* values < .2 were included in the multivariable analyses. A correlation value >0.5 or a variance inflation factor > 2–5 was considered as correlation. Since the variables "place of birth" and "district" correlated highly with the variables for place of vaccination, they were not included. From

the variables that describe access to healthcare, we chose "travel time in rainy season" for inclusion in the regression analyses. The place of vaccination was grouped in different categories to assess seroprotection first on all 3 levels overall, then according to outreach services, and last according to remoteness (HCs grouped according to travel time to DH: <30 min, 30–60 min, >60 min). Binary regressions were performed using a stepwise method for removing variables not associated with the response variable, taking both the *P* value of the variable and the Akaike information criterion of the model into consideration.

RESULTS

Participant Characteristics

A total of 306 children aged 8–23 months were recruited from the CH. Nearly half of the mothers (48%) were aged >30 years and had completed secondary school (45.4%) (Supplementary Table A1). Typically, the child was accompanied by the mother (97.1%), lived <10 km from the nearest HCF (84.3%), was of Tai-Kadai ethnicity (97.7%), and was from a household with an income >2.000.000 Kip (81.4%), corresponding to approximately 232 USD.

A total of 819 children aged 8–28 months were recruited from Bolikhamxay. Typically, children were accompanied by their mothers (80.6%), most of whom were married (97.7%) and of Tai-Kadai ethnicity (84.4%). Most families lived <10 km away from the nearest HCF (72.2%). The characteristics of the participants depended strongly on the place of recruitment. More families were from ethnic minorities at the HC level, their monthly income was less, and more mothers had no education compared to the PH.

Serological Profiles

In Vientiane, at the Children's Hospital, nearly all children had detectable antibodies against diphtheria, tetanus, and Hib, but protective levels varied from 85.1% (tetanus) to 92.5% (diphtheria) and 99.3% (Hib; Table 1). Of participants, 86.9% had anti-HBs ≥10 IU/L, and 57.7% had anti-HBs ≥100 IU/L. Only 36.7% of the children had antipertussis levels ≥22 IU/L, indicating infection or vaccination.

In Bolikhamxay, protective levels of antibodies against tetanus, diphtheria, and Hib were lower by 9.8%, 15.1%, and 27.7%, respectively, compared to the CH. The 9%–28% differences between the children from Vientiane and Bolikhamxay disappeared when the age of the children was taken into account (Figure 1). In addition, seroprotection rates decreased significantly with the age or time since vaccination by 11.8% to 26.4%.

To compare the seroprotection at the different levels of the healthcare system, we age-matched the cohorts and selected only those participants who were vaccinated by the same immunization service with all 3 doses. Under these conditions, there was little difference between children vaccinated at the CH compared to those vaccinated at the PH, except for anti-Hib and the youngest age group for antidiphtheria. Furthermore, children who were vaccinated at HCs did not necessarily have a statistically significant lower overall antibody response than children vaccinated at higher-ranked facilities. However, large differences were observed when the 10 HCs were grouped according to their travel time to the respective DH (Figure 1).

Serological Profiles in Bolikhamxay in 2017 and 2013/2014

In 2017, seroprotection rates in Bolikhamxay seemed to have improved compared to 2013/2014 [4] (Figure 2). Seroprotection increased by more than 20% for antitetanus, antidiphtheria, and anti-HBs(+)/anti-HBc(–) ($P < .0001$), but only by 5% for anti-Hib ($P = .22$). However, 30.9% of the children had antibody levels ≥22 IU/mL against *B. pertussis* in 2013/2014 compared to only 24.9% in 2017. Similar improvements were

Table 1. Overall Percentage of Protective and Detectable Antibody Levels Against Diphtheria, Tetanus, *Haemophilus influenzae* Type B, Pertussis, and Hepatitis B Virus at the Children's Hospital in Vientiane (Aged 8–23 Months) and in Bolikhamxay Province (Aged 8–28 Months)

			Children's Hospital (N = 303–305*)		Bolikhamxay Province (N = 809–817*)	
Antibody	Category	Titer Cutoff	n (%)	CI 95%	n (%)	CI 95%
Antidiphtheria	Protected	≥0.1 IU/mL	282 (92.5)	[89.5–95.4]	631 (77.3)	[74.5–80.2]
	Detectable	≥0.01 IU/mL	304 (99.7)	[99.0–100.3]	773 (94.7)	[93.2–96.3]
Antitetanus	Protected	>0.5 IU/mL	258 (85.1)	[81.1–89.2]	616 (75.3)	[72.4–78.3]
	Detectable	≥0.01 IU/mL	303 (100)	[100]	797 (97.4)	[96.4–98.5]
Anti–*Haemophilus influenzae* type B	Protected	>1 µg/mL	303 (99.3)	[98.4–100.3]	580 (71.7)	[68.6–74.8]
	Detectable	>0.1 µg/mL	305 (100)	[100]	805 (99.5)	[99.0–100]
Anti–hepatitis B surface antigen	Protected	≥10 IU/L	265 (86.9)	[83.1–90.7]	588 (72.0)	[68.9–75.1]
		≥100 IU/L	176 (57.7)	[52.2–63.2]	348 (42.6)	[39.2–46.0]
Antipertussis	Exposure	≥22 IU/mL	112 (36.7)	[31.3–42.1]	200 (24.6)	[21.6–27.6]
	Detectable	≥0.2 IU/mL	291 (95.4)	[93.1–97.8]	673 (82.8)	[80.2–85.4]

Abbreviation: CI, confidence interval.
*Due to sometimes low sample volume, not all samples could be tested.

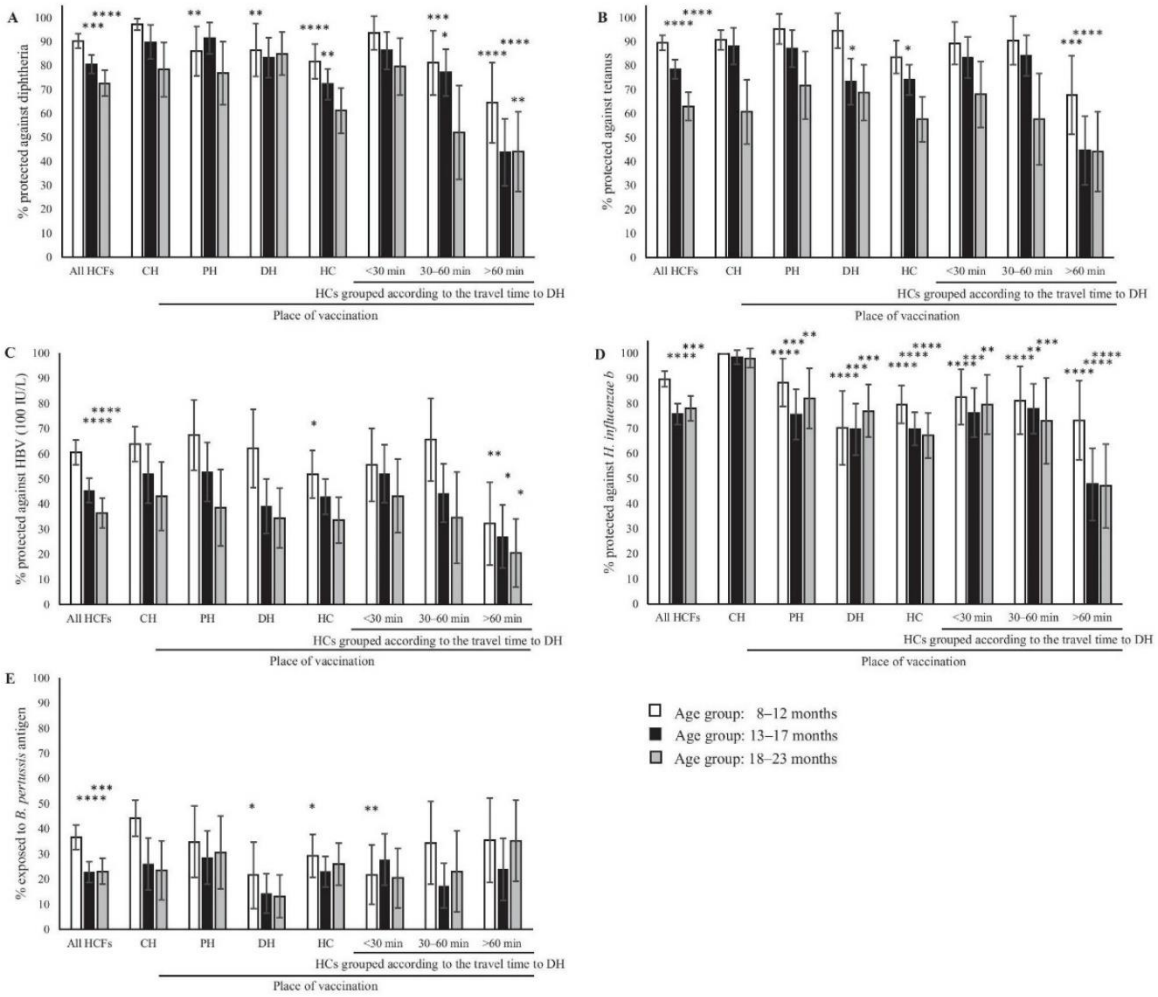

Figure 1. Seroprotection against diphtheria (A), tetanus (B), hepatitis B (C), Hib (D), and pertussis (E) according to place of vaccination in children aged 8–23 months grouped into 3 age ranges at the time of sample collection: 8–12 months, 13–17 months, and 18–23 months. Results are displayed with 95% confidence intervals. Only those children who were vaccinated with all 3 doses by the same immunization service either at a central hospital (CH) in Vientiane, the provincial hospital (PH), the district hospitals (DHs), or the health centers (HCs) were included in the graph. The HCs in the study in Bolikhamxay were further grouped according to travel time to the DH during the rainy season. Immunity was considered protective as described in methods. Seroprotection rates of children aged 13–17 months and 18–23 months at all healthcare facilities combined were compared to the youngest age group (8–12 months). Seroprotection rates between places of vaccination were always compared to the same age group at the CH. Abbreviations: *B. pertussis*, *Bordetella pertussis*; HCF, healthcare facility; HBV, hepatitis B virus; *H. influenzae* b, *Haemophilus influenzae* type b; *H. influenzae*, *Haemophilus influenzae*. *$P \leq .05$, **$P \leq .01$, ***$P \leq .001$, ****$P \leq .0001$.

found when only the villages in the Viengthong district were revisited (Supplementary Figure A2).

When antitetanus and antidiphtheria antibodies were used as a marker of vaccination with the pentavalent vaccine, 94% had detectable levels of antibodies against both tetanus and diphtheria compared to only 83.5% in 2013/2014 in the same age range. The proportion of children protected against tetanus, diphtheria, Hib, and HBV was more than twice as high in 2017 than in 2013/2014 (41.1% compared to 18.5%; Table 2).

In 2017 compared to 2013/2014, the proportion of children who were anti-HBs positive (≥10 IU/L) and anti-HBc negative (Table 3) increased from 31.8% to 69.9% ($P < .0001$). Thus, the number of children who were protected after vaccination increased by more than 2-fold. At the same time, the number of children with an HBV infection decreased by 4-fold (13.18% to 3.11%, $P < .0001$) and the number of HBsAg carriers decreased by 2-fold (1.8% to 0.75%, $P = .15$). In 2017, 77% received the birth dose compared to only 58.2% in 2013/2014. In both years, children with the hepatitis B birth dose were more likely to be seropositive for anti-HBs(+)/anti-HBc(−); ≥10 IU/L ($P = .01$ in 2017 and 0.02 in 2013/2014) and less likely to be negative for both anti-HBs and anti-HBc ($P = .01$ in 2017 and 2013/2014). In

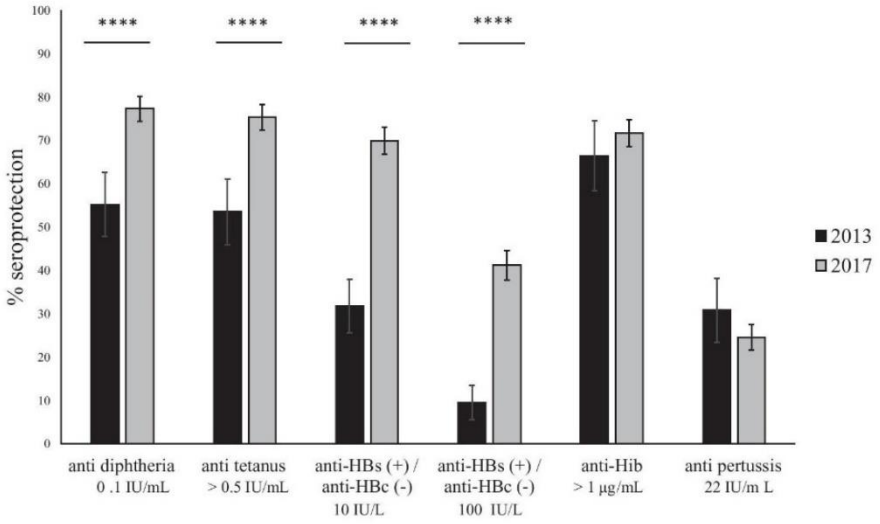

Figure 2. Comparison of the serological profiles of children aged 8–28 months enrolled in Bolikhamxay Province in 2017 and 2013/2014. Seroprotection rates are displayed with 95% confidence intervals. Immunity was considered protective as described in methods. Abbreviations: anti-HBs, anti–hepatitis B surface antigen; anti-HBc, anti–hepatitis B core antigen; anti-Hib, anti–*Haemophilus influenzae* type B. ****$P \leq .0001$.

2017, 2.9% of the children who had received the birth dose were anti-HBc positive compared to 3.78% without the birth dose. In 2013/2014, those proportions were 14.8% and 10.9%, but in both years, the numbers of infections and of chronic carriers were too small to detect a significant impact of the birth dose.

Bivariate Analyses

Bivariate analyses were performed to identify factors associated with a P value less than 0.2 (Supplementary Table A2). Socioeconomic variables showed a strong tendency to correlate with each other due to ethnic and regional disparities. Therefore, in bivariate analyses (Supplementary Table A2), children were less likely to be seroprotected against all the vaccine antigens (except for pertussis) when they were of Hmong-Mien or Mon-Khmer ethnicity, aged >12 months, and vaccinated at remote HCs. In addition, low seroprotection against diphtheria, tetanus, and Hib was associated with poor education, long distance (>10 km or >40 min) to the HCF, or long-term breastfeeding and child age. Participants were more likely to be seroprotected when they lived in Paksan or when their family's income exceeded 500.000 Kip per month. Receiving the hepatitis B birth dose was found to be only slightly associated with anti-HBs titers ≥100 IU/L ($P < .2$). Participants whose mothers stated to have received antenatal care services and more than 3 tetanus vaccination doses during their pregnancy had slightly higher chances to be protected against tetanus ($P < .2$).

Multivariable Analyses

Logistic regression analyses were performed to identify risk factors for vaccine failure in Bolikhamxay (Table 4). In regression analysis, the age of the child and place of vaccination was found to be a strong predictor of seroprotection against tetanus, diphtheria, and HBV. Participants who were aged >12 months and vaccinated at remote HCs were less likely to be protected. In case of diphtheria, Hmong-Mien or Mon-Khmer ethnicity, living more than 10 km from the nearest HCF, and breastfeeding for longer than 6 months were additional risk factors. Participants were less likely to be seroprotected against Hib when they were

Table 2. Relationship Between the Investigated Antibodies in Children From Bolikhamxay in 2017 and 2013/2014

	Protective Antibody Level			
	2017		2013/2014	
Antibody	n/N Tested	%	n/N Tested	%
Antitetanus, antidiphtheria, anti-HBs (≥10 IU/L)	414/812	51.0	27/162	16.7
Antitetanus, antidiphtheria, anti-HBs (≥100 IU/L)	270/812	33.3	15/162	9.3
Antitetanus, antidiphtheria, anti-HBs (≥10 IU/L), anti-Hib	331/806	41.1	23/124	18.5
Antitetanus, antidiphtheria, anti-HBs (≥100 IU/L), anti-Hib	227/806	28.1	13/124	10.5

Abbreviations: anti-HBs, antibodies against hepatitis b surface antigen; Hib, *Haemophilus influenzae* type B.

Table 3. Hepatitis B Virus Serology of Children Tested for the Presence of the Hepatitis B Surface Antigen, Antibodies Against the Hepatitis B Surface Antigen and Antibodies Against the Hepatitis B Core Antigen

	2013/2014							2017						
			Hepatitis B Birth Dose							Hepatitis B Birth Dose				
	All		No		Yes			All		No		Yes		
Hepatitis B Virus Serology	n	%	n	%	n	%	*P* Value[a]	n	%	n	%	n	%	*P* Value[a]
Tested for anti-HBs[b] and anti-HBc:	220	…	92	41.8[c]	128	58.2[c]	…	805	…	185	23[c]	620	77[c]	…
Anti-HBs(+) and anti-HBc(–)	70	31.82	21	22.83	49	38.28	.02	563	69.94	114	61.62	449	72.42	.01
Anti-HBs(–) and anti-HBc(–)	121	55.00	61	66.30	60	46.88	.01	217	26.96	64	34.59	153	24.68	.01
Anti-HBc(+)	29	13.18	10	10.87	19	14.84	.63	25	3.11	7	3.78	18	2.90	.72
Anti-HBs(–), anti-HBc(+):														
HBsAg(–)	11	5.0	3	3.26	8	6.25	…	4	0.50	5	1.08	2	0.32	…
HBsAg(+)	4	1.82	2	2.17	2	1.56	.60[d]	6	0.75	2	1.08	4	0.65	1.00[d]

Abbreviations: anti-HBs, anti–hepatitis B surface antigen; anti-HBc, anti–hepatitis B core antigen; HBsAg, hepatitis B surface antigen.

[a] *P* values for the association of the hepatitis B birth dose with serological profile.

[b] Cutoff used: ≥10 IU/L.

[c] Calculated to the total number of samples tested.

[d] Fisher test.

aged >12 months and when they were of Hmong-Mien or Mon-Khmer ethnicity. The final model with the best fit for the Hib data also comprised the place of vaccination, although the variable was not statistically significant. None of the antipertussis regression models fit well to our data.

DISCUSSION

Seroprotection levels against diphtheria, tetanus, Hib, and HBV (≥10 IU/L) reached 90%–100% for children aged <12 months who were vaccinated at CHs in Vientiane. These results are excellent after recent vaccination in the youngest age group and compare well with results in other countries [23]. The response to the pertussis antigen reached 44% in this setting.

When children were stratified by age, we found a considerable loss in seroprotection against diphtheria, tetanus, and HBV throughout the healthcare system. There was no similar consistent decay in antibodies against Hib and pertussis. The antibody levels at least against the pertussis antigen may also be explained by exposure to the pathogen. The apparent waning of the response to the different antigens may be due either to antibody waning with age or to gradual improvement in management of the cold chain. Overall improvements have been made since 2013 (P. Heimann, Luxembourg Development Cooperation, Laos, personal communication, oral communication, 15 March 2017 and 19 September 2018), but there are no data to quantitate this. In any case, the rapid loss of protection and apparent secondary vaccine failure require further attention.

Interestingly, seroprotection did not necessarily decline with a lower ranking of the facility within the healthcare system. While in absolute terms, seroprotection levels are relatively consistent from the provincial level to the HC level, on the HC level, the distance of the facilities to the respective DH was found to be a strong determinant of seroprotection against hepatitis B, diphtheria, and tetanus. The seroprotection rates are considerably lower in areas served by HCs located more than 60 minutes from its DH. In those remote HCs, 85% of the children who were vaccinated at more remote HCs were vaccinated during outreach sessions, whereas in the more proximate HCs, 68.3% to 72% were vaccinated by outreach. Most children of minorities are served by the more remote HCs, which explains their lower odds to be seroprotected against Hib and diphtheria. Thus, there is a need to specifically address issues that occur within the "last mile" of the vaccine cold chain, that is, the transport of vaccines from DH to HC and the transport from HC to the villages during outreach sessions. A recent study that assessed the cold chain in 2 provinces in Lao PDR reported exposure of vaccines to less than optimal temperatures at the district level [11].

The most encouraging result of our study is a dramatic improvement of seroprotection rates in 2017 compared to our earlier study in 2013/2014 in the same province [4]. This seems to be a reflection of the numerous interventions at the level of vaccine management, infrastructure, and training of staff throughout the cold chain during recent years, with the support and under the supervision of the MoH, LuxDev, UNICEF, the WHO country office and others. However, we cannot exclude that the shift in vaccine manufacturer from Berna/Crucell (Quinvaxem, now Janssen Vaccines Corp.) to the Serum Institute of India may be partially responsible for the enhanced immunogenicity, but we are not aware of similar observations from other countries.

The immune response to the hepatitis B component of the vaccine seemed to have increased by more than 30%. Nevertheless, about one third of the vaccinated children remain unprotected. In addition, about 29.3% of the children with an

Table 4. Logistic Regression on Antidiphtheria Immunoglobulin G, Antitetanus, Anti–hepatitis B surface antigen, and Anti–*Haemophilus influenzae* Type B

			Factors Affecting Antidiphtheria IgG Level				Factors Affecting Antitetanus IgG Level				Factors Affecting Anti-HBs IgG Level				Factors affecting Anti–*Haemophilus influenzae* Type B IgG Level			
Variable	Category (N)[a]	% Protected	OR	95% CI	*P* Value	% Protected	OR	95% CI	*P* Value	% Protected	OR	95% CI	*P* Value	% Protected	OR	95% CI	*P* Value
Socioeconomic factors																	
Travel time to nearest healthcare facility (rainy season), min	<20 (428–431)	79.44	…	…	NS	77.73	….	…	NS	42.79	…	…	…	74.30	…	…	…
	20–40 (183–186)	82.26	…	…	…	78.92	…	…	…	45.41	…	…	…	71.04	…	…	…
	>40 (143–147)	65.99	…	…	…	63.70	…	…	…	41.50	…	…	…	69.23	…	…	…
Vaccinee-related factors																	
Ethnicity of parents/ guardians	Tai-Kadai (619–624)	83.01	1.00	…	…	77.92	…	…	NS	44.55	…	…	NS	76.09	1.00	…	…
	Hmong-Mien + Mon-Khmer (135–138)	52.55	0.36	[0.22–0.59]	**<.0001**	63.50	…	…	…	36.96	…	…	…	56.30	0.44	[0.28–0.70]	**.0002**
Age of mother, y	≤ 20 (56–57)	80.36	…	…	…	77.19	…	…	…	35.09	1.00	…	…	76.79	…	…	…
	20–30 (467–471)	77.66	…	…	…	73.89	…	…	…	42.43	1.32	[0.74–2.42]	.3535	73.12	…	…	…
	>30 (167–169)	76.19	…	…	…	79.17	…	…	…	50.89	1.90	[1.00–3.69]	.0532	71.26	…	…	…
Age of participant, mo	≤12 (187–189)	83.60	1.00	…	…	88.36	1.00	…	…	57.22	1.00	…	…	79.68	1.00	…	…
	>12 (572–575)	75.52	0.50	[0.31–0.79]	**.0040**	71.03	0.29	[0.17–0.47]	**<.0001**	38.61	0.51	[0.35–0.72]	**<.001**	70.19	0.55	[0.36–0.82]	**.0049**
Place of birth	Home (141–144)	75.00	Removed due to correlation			76.92	Removed due to correlation			39.58	…	…	…	70.92	…	…	…
	Health center + district hospital (391–397)	74.87				72.47				44.08	…	…	…	71.36	…	…	…
	Provincial and central hospital + other (221–223)	83.86				79.37				43.89	…	…	…	75.68	…	…	…
Duration exclusive breast-feeding, mo	<6 (512–516)	82.91	1.00	…	…	69.92	…	…	NS	44.19	…	…	…	74.80	…	…	NS
	≥6 (242–246)	66.26	0.59	[0.40–0.87]	**.0075**	77.91	…	…	…	41.06	…	…	…	67.77	…	…	…
Hepatitis B birth dose	Yes (586)		Not applicable				Not applicable			44.88	…	…	NS		Not applicable		
	No (176)									37.50	…	…	…				
Vaccine-related factors																	
District	Paksan (175)	82.86	Removed due to correlation			81.25	Removed due to correlation			45.71	…	…	…	74.86	Removed due to correlation		
	Khamkheut and Vieng-thong (420–428)	72.24				72.54				40.42	…	…	…	69.52			
	Pakkading (159–761)	77.53				75.78				47.80	…	…	…	77.99			
Place of vaccination with health centers grouped according to travel time to district hospital, min[b]	Provincial hospital (176)	82.95	1.00	…	…	81.25	1.00	…	…	45.45	1.00	…	…	74.43	1.00	…	…

Table 4. Continued

Variable	Category (N)[a]	Factors Affecting Antidiphtheria IgG Level				Factors Affecting Antitetanus IgG Level				Factors Affecting Anti-HBs IgG Level				Factors affecting Anti-*Haemophilus influenzae* Type B IgG Level			
		% Protected	OR	95% CI	*P* Value	% Protected	OR	95% CI	*P* Value	% Protected	OR	95% CI	*P* Value	% Protected	OR	95% CI	*P* Value
	District hospital (175–178)	84.09	1.33	[0.75–2.39]	.3296	75.44	0.74	[0.44–1.24]	.255	42.13	0.95	[0.59–1.52]	.832	72.00	0.97	[0.60–1.57]	.9132
	<30 (166–168)	86.90	1.52	[0.83–2.83]	.1793	80.84	0.93	[0.54–1.62]	.802	50.00	1.26	[0.79–2.00]	.3329	78.92	1.41	[0.84–2.36]	.1939
	30 to 60 (126–128)	73.23	0.83	[0.46–1.50]	.5324	80.47	0.94	[0.52–1.70]	.833	47.66	1.15	[0.71–1.88]	.5741	77.78	1.50	[0.86–2.67]	.1574
	>60 (111–114)	50.00	0.41	[0.22–0.76]	**.0050**	51.33	0.22	[0.13–0.37]	**<.0001**	26.32	0.45	[0.26–0.78]	**.0045**	54.95	0.65	[0.37–1.17]	.1527

Data were dichotomized into participants with antidiphtheria IgG greater or less than 0.1 IU/mL, antitetanus IgG greater or less than 0.5 IU/mL, anti-HBs IgG greater or less than 100 IU/L, and anti-Hib IgG greater or less than 1.0 µg/mL. *P*-value < .05 highlighted in bold.

Abbreviations: CI, confidence interval; anti-HBs, anti–Hepatitis B surface antigen antibodies; IgG, immunoglobulin G; NS, not significant; OR, odds ratio.

[a]Total numbers for the categories vary between the analyses of the vaccine components; we could not list them individually due to space limitations.

[b]Each variable describing the place of vaccination was tested individually in the model; only the model with the best fit is reported here. Place of vaccination grouped according to the travel time from health center to the district hospital in the rainy season.

anti-HBs titer of 10–100 IU/L will require a booster dose within 3–6 months after vaccination. Currently, there is no additional booster program for hepatitis B in Lao PDR, leaving many children at risk, albeit at an age when the risk of a chronic infection is considerably lower. The improved response in 2017 was associated with a 4-fold decrease in HBV infection (13.2% in 2013/2014 to 3.1% in 2017).

In addition to geographic limitations, there were several others. Some of the positive serologies may be due to natural infections, at least in cases of diphtheria, *H. influenzae*, and *B. pertussis*. The specific place of vaccination (by outreach service or on site) was based on the parent's recall as it could not be obtained through the HRs. We observed mismatches of vaccination dates in vaccination cards and HRs (manuscript in preparation). Although we can be confident that all children are vaccinated, some may have missed a dose despite documentation.

CONCLUSIONS

Our data show a dramatic improvement in seroprotection rates after routine vaccination with the pentavalent vaccine after interventions to improve vaccine management. While seroconversion rates do not necessarily decline in peripheral facilities, the cold chain continues to be suboptimal particularly in remote areas, thus affecting marginal communities. In addition to strengthening vaccine management with a focus on the last mile of the supply chain, the introduction of a booster dose in remote settings should be considered.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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CHAPTER 5. Seroprevalence of measles and rubella antibodies in vaccinated and unvaccinated infants in the Lao People´s Democratic Republic

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5.1. Contribution statement

The results presented in this study were gained from a collaborative work. The research manuscript presented here is currently under preparation for submission to a peer-reviewed journal. The serum samples and data were collected in the framework of the study described in section **CHAPTER 4**. Laboratory testing was conducted by Dr Xaydalasouk. Data analysis in the study presented here were conducted by Ms Hefele. The initial draft of the manuscript was written by Ms Hefele. All authors wrote and commented on the manuscript. The supplementary material can be found under Appendix **C.d**.

5.2. Bridging section

The importance of using serological data and vaccination dates to monitor and evaluate immunization programmes has been already outlined in context of the pentavalent vaccine in **CHAPTER 4** and applies also to other vaccines in the EPI.

Measles infection remains a serious public health problem in the Lao PDR, despite the fact, that measles vaccination formed part of the initial immunization programme when the EPI was more than 40 years ago. Rubella vaccination was later added to the schedule and at present, both measles and rubella vaccination as given as bivalent MR vaccine (see section **1.4.4**).

The study presented in this chapter utilizes again the data from the cohort described in **CHAPTER 4** and seeks to inform on seroprevalence of measles and rubella antibodies in vaccinated and unvaccinated children. We investigate factors associated with antibody prevalence after vaccination. In addition, the timeliness of children vaccinated with MR was also investigated.

5.3. Abstract

Introduction

Even though measles vaccination was introduced in the Lao PDR in 1984, coverage rates remain consistently low and outbreaks continue to occur frequently. The measles vaccine is given in combination with rubella, which was added in 2011. We investigated the seroprevalence of measles and rubella antibodies in vaccinated and unvaccinated children from Central Lao PDR.

Methods

Antibody titers of 1090 children aged 8-28 months who were vaccinated at different levels of the health care system were assessed by ELISA. Bivariate and multivariable analyses were performed to identify factors affecting seroprotection against measles and rubella. The age at vaccination was calculated using the vaccination dates documented in hospital records and/or on immunization cards.

Results

Among the vaccinated children, 67.5% and 76.4% were double positive/borderline for measles and rubella IgG in Vientiane and Bolikhamxay province respectively, while more children were single positive for rubella than for measles IgG. A high proportion of unvaccinated children at both study sites (24.4% and 38.4%) were positive/borderline for measles and/or rubella. Having received the vaccination <180 days ago, more than two siblings and a mother who is a farmer/labourer was negatively associated with seroprotection. Delayed vaccination was more prominent in lower ranked health facilities.

Discussion

We found a high prevalence of measles and rubella antibodies in unvaccinated children, indicating wide-spread circulation of both viruses and underreporting of cases. The high proportion of susceptible children who received the vaccine suggests problems with vaccine immunogenicity, emphasizing the need for regular evaluations of vaccine efficacy and management. The difference in antibody profiles may suggest higher immunogenicity for the rubella component and/or wider spread circulation of rubella virus. More children vaccinated at lower ranked health facilities were vaccinated later than 12 months compared to children vaccinated in Vientiane. We recommend to strengthen the surveillance of rubella and measles cases by systematically using the case definition for identifying suspected cases and systematic laboratory testing for improved reporting.

5.4. Manuscript

Introduction

With large, recurrent outbreaks throughout many parts of the world, measles vaccination is as important as ever. In 2018, measles accounted for more than 140 000 deaths, most of them children under 5, despite an efficient and safe vaccine available worldwide and for more than 50 years. In November 2019, 413 308 cases were reported globally for that year [148]. Even though most individuals are able to eventually clear measles virus infection and establish life-long immunity, in some patients the infection causes complications such as pneumonia, encephalitis, brain damage, blindness, hearing loss and death [138]. Vaccination against measles is normally administered in combination with rubella and with or without mumps vaccination as bivalent (MR) or trivalent vaccine (MMR). Rubella virus infection causes only mild disease, especially in children. Infection during early pregnancy, however, can cause severe foetal defects, known as congenital rubella syndrome (CRS), miscarriage or stillbirth [150].

The Lao People´s Democratic Republic (PDR) is a land-locked country in South-East Asia with a population of about 7 million people [160]. Vaccination programmes in the Lao PDR are organized along the administrative lines of the health care system: upon arrival in the country, vaccines are stored in a central storage facility in the capital before being distributed to the Central hospitals (CH) in

Vientiane capital or the Provincial hospitals (PH) in each province. From the PH, they are further distributed to the district hospitals (DH) and health centers (HC) [49]. Vaccination services are offered at any of these health care facilities (HCF) or through outreach services.

Measles vaccination was introduced in the Lao PDR in 1984 as part of the National Immunisation Programme (NIP) and rubella vaccination was added in 2011 [91,103]. Currently both vaccinations are given in combination as MR vaccine to children between 9 and 11 months of age. A second MR dose was introduced in 2017 for children between 12 and 18 months of age [142].

In 2019, the estimated coverage rates of vaccination with the first dose of MR reached 69% nationwide, as estimated by WHO/UNICEF[155]. Vaccination coverage rates in the Lao PDR vary widely between districts, with very few districts in 2017 reaching rates above 90% for MR [136]. The mostly low and variable rates throughout the country complicate disease control. Outbreaks of measles in the Lao PDR have been reported in 2012, 2013, 2014 and 2019 [36,122]. Another obstacle to disease control could be low vaccine immunogenicity as reported before in the Lao PDR for other childhood vaccines [28]. Low protection rates and geographical variation suggested problems in vaccine management, particularly in remote areas [28,40]. The timeliness of vaccination may also play a role in disease control [71,125].

Estimates of seroprevalence rates after vaccination with MR in the Lao PDR are rare. A recent study reported prevalence rates of 86.8% and 88.2% for anti-measles and anti-rubella IgG respectively in the age groups from 5 to 21 years, which are the age groups targeted by supplementary immunisation activities (SIA) in 2011. Seropositivity rates were lower in children aged 1 to 2 years born after SIA with about 50% for anti-measles IgG and anti-rubella IgG [36]. In 2015, seroprevalence rates among health care workers were estimated to be 95.4% for measles and 86.2% for rubella antibodies. 11% of the female health care workers were susceptible to rubella infection[10].

In this study, we assess the seroprevalence of measles and rubella antibodies in vaccinated and unvaccinated children, factors associated with antibody prevalence after vaccination and the timeliness of routine MR vaccination in children from Vientiane Capital and Bolikhamxay, a central province.

Methods

Study participants

This study took place in the context of a larger vaccine immunogenicity study [40] which included 1174 children, all of whom had received the three doses of the DTPw-HepB-Hib vaccination, documented in either the hospital record (HR) or yellow cards (YC), from Bolikhamxay province and Vientiane Capital in 2017/18. Due to low serum volumes, not all participants could be tested for measles and rubella

antibodies. 288 children aged 8 to 23 months were included from the 324 participants enrolled at the Children's Hospital in Vientiane Capital. From the 850 participants aged 8 to 29 months who were vaccinated in the PH, three DHs and ten HCs in Bolikhamxay province, 802 were included. During recruitment, the parents/guardians were informed about the study by a health care worker. They could withdraw their participation at any time. Information regarding the participant`s socio-economic background, health care access, vaccination history and location of vaccination was collected with a standardized questionnaire. During data collection at the Children´s hospital, a digitalized version of the questionnaire was used. In Bolikhamxay, the data were collected by a paper-based questionnaire and entered into Epidata. The study was approved by the Lao National Ethics Committee (Reference numbers 033/2017/NECHR, 032/2017/NECHR, 031/2017/NECHR, 056/2017/NECHR) and by the internal ethics review board of the Institut Pasteur du Laos.

The results were compared to antibody levels measured in children of the same age and from the same province during an earlier study from this research group conducted in 2013/14 [28]. In that study, participants between 9-50 months with three documented DTPw-HepB-Hib and a documented MR vaccination were recruited from Bolikhamxay, Vientiane and Khammouane. All samples were tested with the same ELISA kits in the same laboratory. Children recruited in Bolikhamxay aged 8 to 29 months were selected for the comparison to participants in this study.

Vaccination dates

The vaccination history of the participants was recorded from the HR and/or YC. The age of the participants in weeks at the time of the vaccination with measles and rubella was calculated. Vaccination dates in the YC were considered more reliable since they stay with the mothers. Thus, priority was given to the YC to calculate the median age at vaccination. Whenever the YC was not available, the date in the HR was used. In Lao PDR, the MR vaccine is scheduled at 9 to 11 months of age. For the purpose of this study, vaccination between 9 months and 11 months was considered "timely".

Serology

In 2017, venous blood samples (5 ml) were collected from participating children by a health care worker. Serum was separated by centrifugation on the day of collection and stored at 4°C for a maximum of 5 days and then at -20°C for a maximum of two months. Samples were stored afterwards at -80°C at Institut Pasteur du Laos until testing. Commercial ELISA kits (Euroimmun IgG ELISA, Lübeck, Germany) were used to determine IgG antibody levels against measles and rubella virus.

The cut-off values for the antibody levels were based on the manufacturer's instructions: For anti-measles IgG, an antibody titer <200 IU/L was considered negative, a titer between ≥200 to <275 IU/L as borderline and a titer ≥275 IU/L as positive. The detection limit of the anti-measles IgG ELISA was 8 IU/L. An anti-rubella antibody titer <8 IU/ml was considered negative, a titer between ≥8 to <11 IU/ml

as borderline and a titer ≥11 IU/ml as positive for anti-rubella IgG. The detection limit of the anti-rubella IgG ELISA was 0.3 IU/ml.

In logistic regression analysis, borderline samples were considered as positive for anti-measles and for anti-rubella IgG.

Data analysis

Data analyses were conducted using R software [94] with the following packages: epitools[7], car[31], MASS[123], tidyverse[131], rcompanion [67], broom[99].

In Bivariate analyses, Chi-square test and Fisher's exact test were used as appropriate. Odds ratio (OR), 95% confidence intervals (CI) and p-value were calculated. Shapiro-Wilks goodness-of-fit test was used to assess the normality of data and the correlation between two numerical variables was assessed by calculating the Spearman rank correlation coefficient. A p-value<0.05 was considered statistically significant.

Logistic regression with a binomial response variable was performed in order to determine which factors affect seropositivity. Only variables with a p-value<0.2 were included in the binomial generalized linear models (GLMs). A correlation value >0.5 was considered as correlation. Multicollinearity was assessed by deriving the Variance Inflation Factor (VIF) using the vif function (car package). In case of correlation or multicollinearity (VIF >2-5), the variable which was deemed to be less important and/or with the lower impact was removed. Binary regressions were performed using a stepwise method for removing variables that are not associated with the response variable one by one. Both the p-value of the variable and the Akaike Information Criterion (AIC) of the model was taken into consideration during the stepwise regression. The significance of the final model in comparison with the null model was tested using a likelihood ratio test. In addition, the individual effect of the variables in the model was tested by Wald tests. In order to assess the predictive ability of the model, the area under the curve (AUC) was calculated using the "roc" R function.

Results

Participants' characteristics

In Vientiane Capital (n=288), nearly all participants were accompanied by their mother (97.6%) and were of Tai-Kadai ethnicity (97.6%) (Appendix **C.d**, **Supplementary Table 1**). About half of the mothers (52.4%) had completed college or university training, 40% were government employees, 25.4% traders and 26.7% housewives. Most participants lived within 10 km of the nearest HCF (84.4%) and the most common mode of transport to the facility was by private car (76.7%). More than half of the children (59.7%) were less than 12 months old (median age = 9.7 months, ranging from 8 to 23 months).

41.7% of the participants were vaccinated with MR before enrolment. 58.3% of the participants did not have any MR vaccination or were vaccinated at the day of enrolment into the study. For the purpose of this study, the participants who received MR vaccination at the day of the enrolment in the study (n=100; 34.7% of participants in Vientiane) were considered as unvaccinated for their serology but as vaccinated with respect to timeliness of the MR vaccination.

In Bolikhamxay (n=802), 80.5% of the participants were accompanied by their mothers. The majority of the participants belonged to the Tai-Kadai ethnicity and 17.2% belonged to another ethnicity (Appendix **C.d**, **Supplementary Table 1**). Only 15.7% of the mothers had completed a college or university education, but 40.2% had completed primary school and 35.5% had completed secondary school. Nearly half of the mothers were farmers (47.3%) and almost one third (29.6%) were housewives. Most families lived within a 10 km distance of the nearest HCF (72.1%) and most participants were older than 12 months (75.5%) (median age = 16 months, ranging from 8 to 29 months). The majority of the participants from Bolikhamxay were vaccinated with at least one dose of MR according to their vaccination documents (81.3%). Three participants received MR vaccination at the day of the recruitment and were considered as unvaccinated for serology but as vaccinated regarding the timeliness of the MR vaccination. Vaccination dates of 17 (2.1%) participants could not be verified (due to unreadable date, or if it was unclear which date belonged to which vaccination). 25 participants (3.1%) received a second dose of MR according to their immunization cards.

In the entire cohort, 761 participants were older than 11 months and should have already received the MR vaccination (excluding those participants whose vaccination status is unknown). From those, 696 (91.5%) received the MR vaccine.

Prevalence of measles and rubella antibodies

Serological profiles by study site

1090 samples were tested for both measles and rubella antibodies. Overall, 45.4% and 74.3% were positive for measles and rubella antibodies, respectively. 15.5% and 1.3% of the participants were "borderline".

In Vientiane Capital at the Children´s Hospital, 28.5% and 43.1% of the participants were positive for measles or rubella IgG (**Figure 13**). In Bolikhamxay, only half of the participants (51.5%) were positive for measles antibodies and nearly 86% were positive for rubella antibodies.

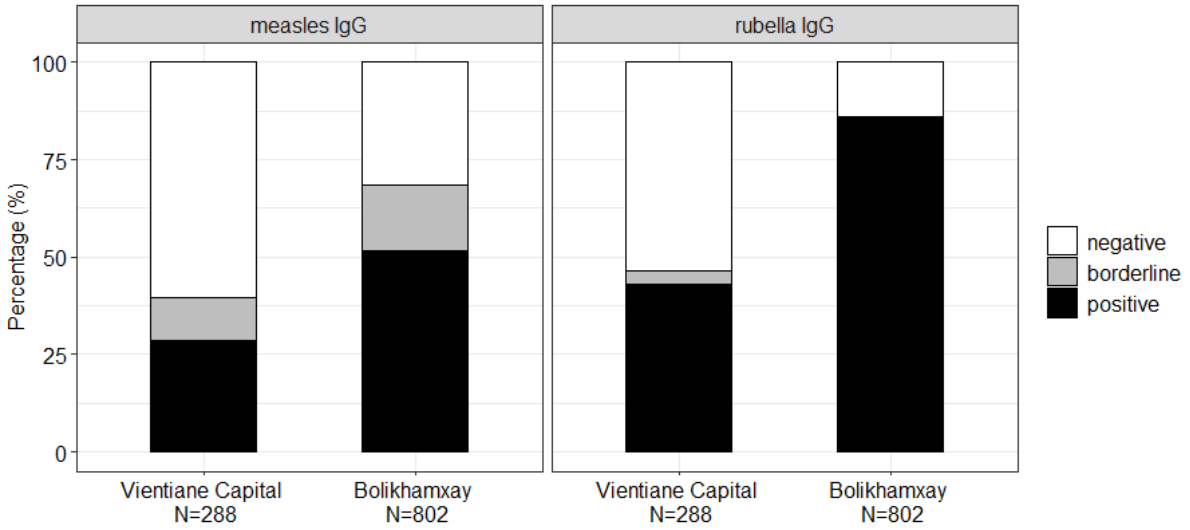

Figure 13 Serological anti-measles and anti-rubella IgG profiles of all participants by recruitment site. N = number of participants.

Serological profiles by vaccination status

In Vientiane Capital, 120 of the participants had documented vaccination against both measles and rubella, excluding those who were vaccinated on the day of enrolment. Among these, only two thirds (67.5%) were positive/borderline for both measles and rubella antibodies (**Table 5**). An additional 29 (24.2%) were positive/borderline for only rubella and 1 (0.8%) was positive/borderline for only measles antibodies. The 168 (58.3%) unvaccinated participants included the 100 participants who were enrolled at the day of their MR vaccination. The majority (75.6%) of the unvaccinated participants were double negative for both antibodies and 15 (8.9%) were double positive/borderline. An additional 17 (10.1%) were single positive/borderline for measles IgG and 9 (5.4%) were single positive/borderline for rubella IgG. From the 100 participants who were enrolled at the day of their MR vaccination, 29% were positive/borderline for either measles or rubella antibodies or for both.

In Bolikhamxay, 83.1% of the participants were vaccinated, but only 498 (76.4%) of them were positive/borderline for both anti-measles and anti-rubella IgG (**Table 5**). An additional 125 (19.2%) were only positive/borderline for anti-rubella IgG and only 1 (0.2%) was positive/borderline for anti-measles IgG (**Table 5**). Among the unvaccinated participants, 82 (61.7%) were double negative. A high proportion were double positive/borderline (25.6%) or only single positive for rubella IgG (12%) or measles IgG (0.8%). The seroprevalence of anti-rubella antibodies in vaccinated children was higher than anti-measles antibodies both in Vientiane (91.7% vs 68.3%) and Bolikhamxay (95.6% vs 76.5%). Seroprevalence of double positives in vaccinated children was slightly higher in Bolikhamxay than in Vientiane Capital (76.4% and 67.5%).

In Bolikhamxay, slightly more unvaccinated children were positive for measles IgG compared to Vientiane Capital (26.3% vs 19.0%). More unvaccinated participants in Bolikhamxay were positive for rubella antibodies than for measles antibodies (37.6% vs 26.3%).

Table 5 Seroprevalence of measles and rubella IgG antibodies in vaccinated and unvaccinated children by recruitment site (participants with unclear vaccination status were not included; N=17)

				Rubella IgG		
				positive & borderline n(%)	negative n(%)	total
VTN	vaccinated (N=120)	Measles IgG	positive & borderline n(%)	81 (67.5)	1 (0.8)	82 (68.3)
			negative n(%)	29 (24.2)	9 (7.5)	38 (31.7)
			total	110 (91.7)	10 (8.3)	**120**
	unvaccinated (N=168)	Measles IgG	positive & borderline n(%)	15 (8.9)	17 (10.1)	32 (19.0)
			negative n(%)	9 (5.4)	127 (75.6)	136 (81.0)
			total	24 (14.3)	144 (85.7)	**168**
BLX	vaccinated (N=652)	Measles IgG	positive & borderline n(%)	498 (76.4)	1 (0.2)	499 (76.5)
			negative n(%)	125 (19.2)	28 (4.3)	153 (23.5)
			total	623 (95.6)	29 (4.5)	**652**
	unvaccinated (N=133)	Measles IgG	positive & borderline n(%)	34 (25.6)	1 (0.8)	35 (26.3)
			negative n(%)	16 (12.0)	82 (61.7)	98 (73.7)
			total	50 (37.6)	83 (62.4)	**133**

VTN = Vientiane Capital; BLX = Bolikhamxay

Serological profiles in Bolikhamxay in 2017 and 2013/2014

The serologies of the 652 vaccinated participants from 2017/18 were compared to results from vaccinated participants in the same age range (8 to 28 months, n = 155) who were enrolled in a vaccine immunogenicity study in 2013/14 [28]. In both studies, the proportion of male and female participants was similar (53.6% and 53.1% male participants). In the 2013/14 study the participants were on average about 3 months older (mean = 19.5 months, median = 19 months) than participants included in the 2017 study (mean = 16.2 months, median = 16 months). The time since MR vaccination with was 2 months longer (0 to 22.9, mean = 9.2, median = 8.9 months) for participants in the 2013/14 study as compared to the 2017 study (0 to 19, mean = 7.4, median = 7.2 months).

In 2017, the anti-rubella seroprevalence was higher following vaccination compared to 2013/14 (95.5% vs 82%) (**Table 6**). While the anti-measles seroprevalence also increased from 66% in 2013/14 to 76.5% in 2017, it remains sub-optimal. Among vaccinated children in both studies, only 59.6% in 2013/14 and 76.4% in 2017 were seropositive to both anti-measles and anti-rubella.

Table 6 Seroprevalence of measles and rubella IgG antibodies in vaccinated participants included in the studies in 2013/14 and 2017 in Bolikhamxay province.

				Rubella IgG		
				positive & borderline n(%)	negative n(%)	total
Bolikhamxay 2013/14	Vaccinated (N=155)	Measles IgG	positive & borderline n(%)	92 (59.6)	10 (6.5)	102 (65.8)
			negative n(%)	36 (23.2)	17 (11.0)	53 (34.2)
			total	128 (82.6)	27 (17.4)	**155**
Bolikhamxay 2017	Vaccinated (N=652)	Measles IgG	positive & borderline n(%)	498 (76.4)	1 (0.2)	499 (76.5)
			negative n(%)	125 (19.2)	28 (4.3)	153 (23.5)
			total	623 (95.6)	29 (4.5)	**652**

Factors associated with measles and rubella antibody prevalence in vaccinated participants from Bolikhamxay province

652 participants in Bolikhamxay had received the MR vaccination. Factors associated with seroprevalence of double positive/borderline measles and rubella antibodies after vaccination were investigated by bivariate and multivariable analysis (**Table 7**). All positives for measles were also positive for rubella, except for one participant. In bivariate analysis, participants were more likely to be positive/borderline for measles and rubella antibodies when their mothers had a higher socio-economic status (i.e. being traders or employees or had received a higher education) and when the children were born at a DH or PH as compared to at home or at a HC. Surprisingly, longer time since vaccination (more than 180 days ago) was also associated with higher seroprevalence. Mon-Khmer or Hmong-Mien ethnicity, having more than 2 siblings, living more than 10 km from the nearest HCF or having been vaccinated at a HC were negatively associated with being double positive/borderline for both anti-rubella and anti-measles IgG.

All variables that were associated with the outcome with $p < 0.2$ were included in the multivariable regression model. The variables “age of child” and “time since vaccination” correlated with each other and the model was fitted with each variable separately. After stepwise backwards regression, participants with more than two siblings, whose mothers were farmers or labourers or who were vaccinated < 180 days ago were less likely to be seropositive. In addition, having been vaccinated at a HC or living more than 10 km from the nearest HCF were also retained in the final model, but were not significant. The fit of the overall model in comparison to the null model was significant (p-value < 0.0001, AUC = 68.3%, Pseudo-R^2=15.2%).

Table 7 Risk factor analysis for being double positive or borderline for both anti-measles and anti-rubella IgG in vaccinated participants in Bolikhamxay

Variable	Categories	Number of rubella & measles IgG positive/borderline per total number (%)	Bivariate analysis		Multivariable analysis	
			OR [95% CI]	p-value	OR [95% CI]	p-value
Age of the mothers	≤ 20	31/45 (68.9)		NS		
	> 20 - ≤ 30	297/397 (74.8)				
	> 30	122/156 (78.2)				
	NA	48/54 (88.9)				
Ethnicity	Tai-Kadai	436/556 (78.4)	ref			
	Mon-Khmer & Hmong-Mien	62/96 (64.6)	0.5 [0.32-0.8]	0.006		
Occupation of mother	Farmer & Labourer	213/311 (68.5)	ref			
	Trader, gov. employee, priv. employee	285/341 (83.6)	2.34 [1.61-3.4]	< 0.0001	1.85 [1.2-2.88]	0.006
Level of education of mother	None & primary school	222/308 (72.1)	ref			
	Secondary school & University	276/344 (80.2)	1.57 [1.09-2.26]	0.016		
Antenatal care	no/unknown	45/63 (71.4)		NS		
	yes	453/589 (76.9)				
Tetanus vaccination during pregnancy	yes	412/543 (75.9)		NS		
	no	86/109 (78.9)				
Houshold members	< 6	261/349 (74.8)		NS		
	≥ 6	237/303 (78.2)				
Household income	≤ 1,000,000 Kip	197/265 (74.3)		NS		
	> 1,000,000 Kip	301/387 (77.8)				
Distance to nearest HCF	< 10 km	379/479 (79.1)	ref			
	≥ 10 km	119/173 (68.8)	0.58 [0.39-0.86]	0.009	0.67 [0.44-1.02]	0.061
Age of child	≤ 12 months	66/103 (64.1)	ref		Correlated with time since MR vaccination	
	> 12 months	432/549 (78.7)	2.07 [1.32-3.25]	0.002		
Sex of child	male	272/350 (77.7)		NS		
	female	226/302 (74.8)				
Duration of breastfeeding	≤ 6months	339/446 (76)		NS		
	> 6 months	159/206 (77.2)				
Number of siblings	< 2	183/221 (82.8)	ref			
	≥ 2	315/431 (73.1)	0.56 [0.37-0.85]	0.006	0.52 [0.34-0.8]	0.003
Place of birth	At home or HC	170/249 (68.3)				
	PH, DH, CH	328/403 (81.4)	2.03 [1.41-2.93]	< 0.001		
Hepatitis B birth dose	yes	395/514 (76.8)		NS		
	no	103/138 (74.6)				
Time since vaccination	< 180 days	171/246 (69.5)	ref			
	≥ 180 days	320/395 (81)	1.87 [1.29-2.71]	0.001	1.93 [1.32-2.83]	0.001
	NA	7/11 (63.6)				
Place of vaccination	PH/DH	263/314 (83.8)	ref			
	HC	235/335 (70.1)	0.46 [0.31-0.67]	< 0.0001	0.66 [0.42-1.02]	0.061
	NA	0/3(0.0)				

NS = not significant; HC = health center; DH = district hospital; PH = provincial hospital; CH = central hospital; HCF = health care facility

Timeliness of MR vaccination

The majority of participants in Vientiane Capital (76.4%) and in Bolikhamxay (81.7%) were vaccinated as documented by vaccination records (including those participants who were vaccinated at the day of recruitment).

At both study sites, the median age at vaccination with MR was 10 months, ranging from 9 months to 23 months in Vientiane and from 3 months to 21 months in Bolikhamxay (Appendix **C.d**, **Supplementary Table 2**). In Bolikhamxay the median age at vaccination was similar irrespective of the health facility level. At each study site most participants were vaccinated with MR between the age of 9 and 11 months of age (**Table 8**), however, the proportion of participants vaccinated after 11 months of age increased from 6% at the CHs to 33.7% - 46.7% in lower ranked HCFs.

Among the participants recruited in Vientiane at the CH, the median time since vaccination was 83 days (corresponding to approximately 3 months; ranging from 0 to 455 days), while it was 216 days (approximately 7 months; ranging from 0 to 570 days) among the participants in Bolikhamxay province.

Table 8 Age at vaccination by health care level

	Health care level					
Age at vaccination[1]	CH (N=217) n(%)	PH (N=157) n(%)	DH-facility (N=92) n(%)	DH-outreach (N=61) n(%)	HC-facility (N=60) n(%)	HC-outreach (N=270) n(%)
< 9 months	0 (0.0)	4 (2.6)	2 (2.2)	0 (0.0)	1 (1.7)	8 (3.0)
9-11 months	201 (92.6)	141 (89.8)	57 (62.0)	35 (57.4)	31 (51.7)	158 (58.5)
12-18 months	13 (6.0)	12 (7.6)	31 (33.7)	26 (42.6)	28 (46.7)	100 (37.0)
> 18 months	3 (1.4)	0 (0.0)	2 (2.2)	0 (0)	0 (0.0)	4 (1.5)

[1]Participants were removed from the table when the place of vaccination was unkown or when the calculated time value was negative (since it indicates a mistake made in the vaccination records) (n = 15)
CH = Central Hospital, PH = Provincial Hospital, DH = District Hospital, HC = Health Center.

Discussion

The present seroprevalence study included both unvaccinated children as well as children with documented MR vaccination. Thus, we were able to evaluate and compare the prevalence of measles and rubella in children attending typical health facilities in rural Bolikhamxay province with those attending central hospitals in Vientiane capital. We found that a very high percentage of unvaccinated children had already antibodies against measles in both the rural and urban location. In rural Bolikhamxay slightly more unvaccinated children were already seropositive for anti-measles IgG by the time of enrolment than in Vientiane Capital (26.3% versus 19%). The high prevalence of measles is surprising, since only 8 and 3 measles cases have been reported in the Lao PDR in 2016 and in 2017 [152]. Our cohort included children 8 to 29 months. Thus some of the younger children, e.g. below the

age of 10 months may still have had persisting maternal antibodies. However, in an unpublished study, we found that by 8 months of age virtually all children had lost their maternal antibodies. Thus, maternal antibodies cannot explain the apparent high prevalence of measles in this cohort. If this reflects the true incidence of measles in these children in these two locations, the disease is still highly prevalent and highly underreported.

The prevalence of rubella antibodies was similar to that of measles in Vientiane (14.3% vs. 19%), but considerably higher in Bolikhamxay than in Vientiane (37.6% vs. 14.3%) and compared to measles in Bolikhamxay (37.6% vs. 26.3%). This high seroprevalence of rubella in both locations can again not be explained by persisting maternal antibodies, since these are lost much earlier (unpublished results). Since rubella vaccination was only introduced in 2011, i.e. only 6 years before this study, the rubella virus may still circulate much wider than the measles virus. With only 10 rubella cases reported during the year of this study in the Lao PDR, rubella, similar to measles seems highly underreported [152].

One reason for the persisting high incidence of measles and rubella could be a low efficacy of the MR vaccine and the weak response/seroconversion of children to the two components of the vaccine. Indeed, in our sub-cohort of children with documented MR vaccination only 68.3%-76.5% had antibodies against measles. In contrast more than 90% were anti-rubella seropositive (91.7%-95.6%). This may reflect the higher immunogenicity of the rubella component of the vaccine and/or a higher circulation of the rubella virus in this population. Both components may differ in terms of stability as shown in a study in the Lao PDR in 2018 [36] in which the measles component of the vaccine was found to be more heat-sensitive. The anti-measles and anti-rubella seroprevalence is only slightly higher in Bolikhamxay than in Vientiane (measles: 76.5% vs 68.3%; rubella 95.6% and 91.3%), suggesting either higher natural infection in Bolikhamxay or a higher immunogenicity of the vaccine.

The comparison of the anti-measles and anti-rubella seroprevalence between 2017 and 2013/2014 in rural Bolikhamxay showed a considerable difference. Between the two studies, the anti-rubella seroprevalence increased from only 82 to 95.5%. While the anti-measles seroprevalence also increased from 66 to 76.4%, it nevertheless remains below the 90-95% needed to ensure herd immunity [77]. In 2017 and 2013/2014 only 59.6% (92/155) and 76.4% (498/652) were double seropositive for both anti-measles and anti-rubella, because of the low anti-measles seroprevalence. The increased seroprevalence over the years may be due to a better vaccine response through improved vaccine management and is in line with the approximately 20% increase in protection rates against diphtheria, tetanus and hepatitis B in the current cohort compared to 2013/14 [28,40]. Overall, considerable improvements in health care and health infrastructure have been made since 2013 (P. Heimann, Luxembourg Development Cooperation, Laos, personal communication, oral communication, 15 March 2017 and 19 September 2018). It should be noted, that the vast majority of the children in our study received only one dose of

the MR vaccine. A second dose of the MR vaccine was introduced in 2017 for children and may also improve seropositivity rates in the future [142].

In our study only children were included who had received all three doses of the pentavalent DTPw-HepB-Hib vaccine. This represents a significant selection bias in favour of those with access to vaccination services. Among the participants older than 11 months, 91.5 % had received the MR vaccine. Compared to the general population of children in Bolikhamxay, this is certainly an overestimation of vaccine coverage. In 2019, the nationwide vaccination coverage with MR was estimated to be 69% [157]. Since problems with health records and management in the Lao PDR have been previously observed by us [39] and others [112], we cannot exclude the possibility that some of the documented vaccinated children did not receive their vaccination. For the same reason, we also cannot exclude the possibility that children without a record did indeed receive the MR vaccination, which could explain the high seroprevalence rates in presumably unvaccinated children.

In logistic regression, having more than two siblings, a mother who is a farmer or labourer and having received the vaccine less than 180 days ago were independently associated with being less likely to be seronegative against both measles and rubella. The distance to the nearest HCF and vaccination at a HC also seemed to play a role and were retained in the best fitting logistic regression model, but these variables were not significant. In our previous study [40], the place of vaccination was strongly associated with vaccine-induced seroprotection against diphtheria, tetanus, and hepatitis B. These finding warrant further investigation and underline the need to strengthen vaccine management on lower ranked levels of the health care system. Interestingly, in the current study, participants were more likely to be positive if they had received their MR vaccination more than 180 days prior to sample collection. This finding could be indicative of continued exposure to circulating measles virus. Unfortunately, our study only covered a limited age range and we could not further investigate the antibody dynamics in this cohort.

The median age at vaccination was found to be similar at both study sites, with 10 months at the central hospitals in Vientiane and 10 to 11 months in Bolikhamxay. In Bolikhamxay, MR vaccination was mostly given between the age 9 to 12 months (89.8%). However, the proportion of participants vaccinated after 12 months of age increased from 6% at the CHs to 33.7% - 46.7% in lower ranked HCFs. We have previously observed vaccination delays on lower levels of the health care system for the pentavalent vaccine [39]. Vaccination coverage and vaccination timeliness are separate issues but they are connected: Delays in routine vaccination increase the risk of missed opportunities. Delayed vaccination also increases the window of disease susceptibility facilitating disease outbreaks.

Limitations

Besides the geographic limitations, it is not possible to differentiate between natural and vaccine-induced antibodies, which complicates the interpretation of our findings. The specific place of vaccination (by outreach service or on site) was based on the parents' recall since it is not recorded in the vaccination documents. We observed mismatches of vaccination dates in vaccination cards and HRs before [39] and cannot exclude that not all participants with a vaccination date were truly vaccinated.

Conclusion

In this study, we found a high prevalence of anti-measles and anti-rubella antibodies in unvaccinated children at both study locations, which may be indicative of wide-spread circulation of both viruses and severe underreporting of measles and rubella cases. We recommend to strengthen the surveillance of rubella and measles cases by systematically using the case definition for identifying suspected cases and systematic laboratory testing for improved reporting. At this stage, where most countries are at a case-based surveillance, it is important to have a better understanding of the sensitivity of the MR surveillance system in Laos. The difference in measles and rubella antibody prevalence in vaccinated children reflects higher immunogenicity of the rubella component of the vaccine and/or a more active circulation of rubella virus. Compared to a previous study, the percentage of double positive vaccinated children increased, but the response to the measles component of the vaccine remains substantially lower than the 90-95% threshold required for establish herd immunity. These results suggest a thorough evaluation of vaccine management is needed. More children vaccinated at lower ranked health facilities were vaccinated later than 12 months compared to children vaccinated in Vientiane, underlining the need to address the gaps in health care access.

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CHAPTER 6. Timeliness of immunisation with the pentavalent vaccine at different levels of the health care system in the Lao People's Democratic Republic: a cross-sectional study

6.1. Contribution statement

The study presented in this section was published as:

Lisa Hefele, Sengdavanh Syphan, Dalouny Xayavong, Anousin Homsana, Daria Kleine, Phetsavanh Chanthavilay, Phonethipsavanh Nouanthong, Kinnaly Xaydalasouk, Outavong Phathammavong, Somxay Billamay, Anonh Xeuatvongsa, Daniel Reinharz, Antony P. Black, Claude P. Muller. 2020. *Timeliness of immunisation with the pentavalent vaccine at different levels of the health care system in the Lao People's Democratic Republic : A cross-sectional study*. PLoS One 1–15. https://doi.org/10.1371/journal.pone.0242502

Ms Hefele supervised sample collection, data acquisition and laboratory testing; conducted data cleaning, primary and final data analysis, summarized the findings, wrote the initial draft and wrote and edited on the manuscript. An author contribution statement signed by all authors was supplied separately. The supplement material can be found in Appendix **C.e**.

6.2. Bridging section

Apart from coverage rates and seroprotection levels, the timeliness of vaccination can also play a major role in the evaluation of vaccination programmes. If vaccinations are given later than indicated in the schedule, the risk of getting infected before the vaccination may rise, affecting both herd immunity and the health of the individual. Premature vaccination or vaccination doses given in too little time intervals (in case of multiple dose vaccinations) may not be protective.

Information regarding the timeliness of specific vaccination in the Lao PDR is scarce. Building on the data from the cohort described in **CHAPTER 4**, the timeliness of the immunization with DTPw-HepB-Hib was investigated. In addition, possible predictors for the timely completion of the vaccination course were assessed by bivariate and multivariable analyses.

6.3. Publication

PLOS ONE

RESEARCH ARTICLE

Timeliness of immunisation with the pentavalent vaccine at different levels of the health care system in the Lao People's Democratic Republic: A cross-sectional study

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Data Availability Statement: Due to ethical restrictions and participant confidentiality, the data set cannot be made publicly available. However, the data can be requested from Institut Pasteur du Laos, after obtaining permission from the National Ethics Committee for Health Research (NECHR) in the Lao PDR. The contact person for the NECHR in the Lao PDR is Dr Khampheng Phongluxa (Tel: +856-21 250670-207 and 208; Email: khampheng_p@hotmail.com; Website: http://www.laohrp.com). The Institut Pasteur du Laos can be

Abstract

Background

The timely administration of vaccines is considered to be important for both individual and herd immunity. In this study, we investigated the timeliness of the diphtheria-tetanus-whole cell pertussis-hepatitis B-*Haemophilus influenzae* type b (pentavalent) vaccine, scheduled at 6, 10 and 14 weeks of age in the Lao People's Democratic Republic. We also investigated factors associated with delayed immunization.

Methods

1162 children aged 8–28 months who had received the full course of the pentavalent vaccine at different levels of the health care system were enrolled. Vaccination dates documented in hospital records and/or immunisation cards were recorded. Age at vaccination and time intervals between doses were calculated. Predictors for timely completion with the pentavalent vaccine at 24 weeks were assessed by bivariate and multivariable analyses.

Results

Several discrepancies in dates between vaccination documents were observed. In general, vaccination with the pentavalent vaccine was found to be delayed, especially in health care

contacted through the website (https://www.pasteur.la/contact/), by phone (Tel: +856-21 285321) or by email through Antoine des Graviers (a.desgraviers@pasteur.la).

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Competing interests: Dr. Nouanthong has been a member of the independent National Immunization Technical Advisory Group in Lao PDR since August 2017. The other authors declared no conflict of interest. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

settings below the provincial hospital level. Compared to the central hospital level, less participants who were vaccinated at the district/health center level received the third dose by 16 (48% at the central hospital level vs. 7.1% at the district and 12.4% at the health center level) and 24 weeks of age (94.4% at the central hospital level vs 64.6% at the district-outreach and 57.4% at the health center level) respectively. In logistic regression analyses, lower education level of the mother as well as vaccination by outreach service, were independently associated with delayed completion of vaccination.

Conclusion

We observed a general delay of vaccination, especially at lower ranked facilities, which correlated with indicators of poor access to health services. This highlights the need for further improving health equity in rural areas. Age-appropriate vaccination should become a quality indicator for the national immunization programme. In addition, we recommend further training of the health care staff regarding the importance of reliable documentation of dates.

Introduction

The timely administration of vaccines is considered to be important for both individual and community immunity. Studies in the UK [1], China [2], the USA [3] and lower-income countries such as Senegal [4], Sri Lanka [5], Malawi [6] and India [7, 8] have investigated the impact of timeliness on vaccination. Delayed vaccinations of children may increase the risk of infection before vaccination, compromising the success of the intervention as well as herd immunity. On the other hand, vaccinations given too early or without sufficient interval between the doses may not be fully protective [1].

In the Lao People´s Democratic Republic (PDR), the expanded programme on immunization (EPI) is one of the most successful public health programmes and routine vaccination coverage has increased for more than ten years. Currently, the National Immunization Programme in Lao PDR includes vaccines against tuberculosis, measles, rubella, diphtheria, tetanus, pertussis, *H. influenzae* type b, polio, pneumococcal disease and Japanese encephalitis. Vaccination of children with vaccines included in the National Immunization Program is free of charge.

The estimated coverage for the 3rd dose of the pentavalent diphtheria-tetanus-pertussis-hepatitis B-*Haemophilus influenzae* type b vaccine (pentavalent vaccine) was 84% [9] nationwide in 2018 and 84.1% specifically in Bolikhamxay province in 2017 [10]. However, it was reported in 2012 that only a small number of children had received all 3 doses of the pentavalent vaccine by the recommended age of 4 months in Lao PDR [11]. While there are clear national recommendations for delivery of the pentavalent vaccine doses in Lao PDR, little is known at which age children actually receive the vaccine within the National Immunization Program and whether this may impact the immune response. The pentavalent vaccine is scheduled at 6, 10 and 14 weeks of age. Vaccination schedules vary between countries. Vaccination with the DTP-containing vaccine in Thailand follows a 2-4-6 months schedule with a booster vaccination at 4 years [12]. The United States of America follow the same schedule for the primary vaccination but include a booster at 15 months [13] while some European countries such as Germany, Luxembourg and Belgium follow a 2-3-4 months schedule but differ with respect to the timing of the booster dose [14]. Generally, the minimum age of vaccination for the DTP-containing vaccine is six weeks and the recommended spacing between the doses

is four weeks. Vaccination doses given too early after the first one may result in an impaired vaccine response [13]. However, there is no consensus on definitions for vaccination timeliness [15].

Achieving a high coverage with the (monovalent) hepatitis B birth dose is important to prevent mother-to-child transmission of Hepatitis B. Even though the birth dose was introduced in Lao PDR in 2003, the nationwide coverage was only 55% in 2018 [16]. Therefore, the birth dose coverage continues to be an important issue in Lao PDR.

In Lao PDR, central hospitals (CH) in Vientiane Capital represent the highest level of the health care system, followed by provincial hospitals (PH), district hospitals (DH) and health centers (HC) [17]. CHs are tertiary care facilities, PHs and DHs provide health promotion, disease prevention and treatment services, but are limited in capacity and expertise. HCs provide only basic medical services, including immunization services and maternal, new-born and child health services [18, 19]. For vaccinations, the parents tend to rely on the nearest health care facility. Although vaccination for children is free, not all families can afford out-of-pocket payments for travel and sustain the loss of a day's work. Typically, health care facilities (HCF) such as HC or DH provide immunization services to several villages within a flexible radius. Vaccinations are registered in the yellow child vaccination card, which stays with the family, and the hospital records, which can consist of one registry book at the mother and child department but also includes the vaccination books of the EPI team, typically one vaccination book for each village. Regardless of where the vaccination takes place, the children are usually listed in the book of the home village and in case of outreach vaccination, the books are taken to the villages. The hospital records as well as vaccination cards are standardized and provided by the health offices.

The primary objective of this study was to investigate the timeliness of vaccination with the three doses of the pentavalent routine childhood vaccine in fully vaccinated children in Bolikhamxay province and Vientiane Capital. In addition, the timeliness of Hepatitis B birth dose was assessed. For this purpose, the dates of vaccination as recorded in the vaccination card and hospital records were compared and the proportion of children that received the vaccination with delay was estimated. Furthermore, we investigated risk factors associated with delayed vaccination.

Participants and methods

Participants

The study took place in the context of a larger vaccine immunogenicity study in Bolikhamxay province and in Vientiane capital in 2017/18 (see S1 File) [20]. All participants had received the full course of the pentavalent vaccine, documented in either the hospital records or vaccination card. In Vientiane Capital, 319 children aged 8 to 23 months and their parents/guardians who visited the Children´s Hospital for unrelated health reasons or Measles and Rubella vaccination were enrolled. Bolikhamxay is a central province only about 150 km away from the capital, on the highway to the South. Bolikhamxay comprises 291 villages with 53 964 households including 304 000 inhabitants in 7 districts [21]. In Bolikhamxay province, 843 children aged 8 to 28 months were recruited, who were vaccinated in the PH, three DHs and ten HCs. Before the start of the sample collection, the study was explained to the head of the participating village and to the parents of the participants by a health care worker. All parents/guardians signed the informed consent form and could withdraw their participation at any time. A standardized questionnaire was designed to collect information about the participant´s socio-economic background, access to health care, history and location of vaccination. The detailed information collected by the questionnaire can be found in S1 Table in S1 File of the

previously published study. Vaccination histories were verified and confirmed in the hospital records at the HCF, if available, as well as on the vaccination card. The data was double entered into Epidata [22] independently before data analysis. Serum samples were collected from participating children to assess antibody levels. The study was approved by the Lao National Ethics Committee (Reference numbers 033/2017/NECHR, 032/2017/NECHR, 031/2017/NECHR, 056/2017/NECHR) and by the internal ethics review board of the Institut Pasteur du Laos.

Vaccination dates

During recent years, the vaccination schedule expanded with the inclusion of new vaccines and/or additional doses, and the layout of vaccination cards and hospital records were gradually adapted. During the review of the vaccination history, we came across different formats and versions of the vaccination card and hospital records. Major changes in format were introduced with the pneumococcal vaccine (PCV13), the inactivated polio vaccine (IPV), the Japanese encephalitis vaccine (JEV) and the second dose of the measles-rubella vaccine (MR). Not all facilities used the latest date version of the hospital records or vaccination cards. However, since the pentavalent vaccine was introduced already in 2009/10, it was included in all versions of the vaccination card and the hospital records inspected in this study [23].

The vaccination history of the participants was recorded from the hospital records and/or vaccination cards. The age of the participants in weeks at the time of the vaccination with the pentavalent vaccine and with the hepatitis B birth dose was calculated based on the birthdate and the date of the vaccination. Since there is no consensus-definition of "timely vaccination" in lower middle-income countries, as discussed in a review [15], we used both continuous and categorical measures. The age when receiving each vaccine dose was defined as: "timely" when between 6–7 weeks for dose 1 (pentavalent 1), 10–11 weeks for dose 2 (pentavalent 2) and 14–15 weeks for dose 3 (pentavalent 3) (Table 1). When the participant was older or younger, the vaccination was considered to be "earlier" or "later" than recommended. An interval of 4 weeks is recommended between each dose and intervals shorter than 4 weeks or longer than 5 weeks were considered "shorter" or "longer" than recommended. The National Immunization Programme of the Lao PDR recommends the primary vaccination with the DTP-containing vaccine is completed at 14 weeks and the WHO guidelines specify completing the vaccination course latest at 6 months (24 weeks) of age [24]. In this study, we considered 16 weeks (in order to give some time margin) and 24 weeks (the latest WHO recommendation for completeness) as cut-off for"early timely" and "late timely" completion of vaccination with the pentavalent vaccine (Table 1). The proportion of participants that had received the pentavalent vaccine by 16 and 24 weeks at each health care level was compared to the level of the CH. Furthermore, we used 24 weeks as a cut-off in order to identify factors associated with the timely completion of the schedule or not.

In order to detect irregularities in the vaccination documents of participants who had both the hospital records and vaccination card available, the time discrepancy between documented vaccination dates was obtained by subtracting the vaccination date in the vaccination card from the

Table 1. Definitions for timeliness of vaccination with the pentavalent vaccine.

Vaccine dose	Recommended age	Definition of timeliness	Explanation
Pentavalent 1	6 weeks	6–7 weeks	Adherence to vaccination schedule
Pentavalent 2	10 weeks	10–11 weeks	Adherence to vaccination schedule
Pentavalent 3	14 weeks	14–15 weeks	Adherence to vaccination schedule
		by 16 weeks	Early completion of full vaccination with the pentavalent vaccine
		by 6 months	Late completion of full vaccination with the pentavalent vaccine

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vaccination date in the hospital records. Vaccination dates in the vaccination cards were considered more reliable as the cards stay with the mothers and are filled in on the day of the vaccination. Hospital records can also be entered at a later time point and problems with medical documentation has been reported in Lao PDR before [25]. Therefore, we gave priority to the vaccination cards to calculate the median age at vaccination and the intervals between vaccinations. Whenever the vaccination card was not available, the date in the hospital records was used.

Laboratory analysis

The serum samples were analysed by commercial ELISA kits as described elsewhere [20]. Protective immunity was considered if the participants had an anti-diphtheria titer $\geq$0.1 IU/ml, an anti-tetanus titer >0.5 IU/ml, an anti-hepatitis B titer >10 IU/L and an anti-*Haemophilus influenzae* type b (Hib) titer >1.0μg/ml. A titer $\geq$22 U/ml was used as indication of exposure to the vaccine antigen for *B. pertussis*.

Statistical analysis

Data analyses were conducted using R software (version 3.5.3) [26] with the following packages: "tidyverse" [27], "lubridate" [28], "MASS" [29], "rcompanion" [30], "lmtest" [31], "car" [32], "epitools" [33], "ggplot2" [34], "survival" [35], "survminer" [36] and "pROC" [37].

Survival analysis by the Kaplan-Meier method was performed to present timeliness of vaccination for each of the three doses of pentavalent vaccine at any given age (S3 Fig), a method suggested by Lauberau et al. (2002) [38]. For the Kaplan-Meier curve, we used the age at vaccination as based on the date in the vaccination card, and if there was no card available, we used the date in the hospital records. Participants for which the calculated age at vaccination was negative (date of vaccination before date of birth, a documentation error) were excluded.

In order to assess whether any of the socio-demographic or vaccination related factors are associated with the completion of primary vaccination with the pentavalent vaccine by 24 weeks (6 months) of age, Chi-square test and Fisher's exact test were performed. Odds ratio (OR), 95% confidence intervals (CI) and p-value were calculated. We performed logistic regression analysis in order to investigate the association between the binomial response variable (completion of vaccination by 24 weeks) and socio-economic or vaccination-related factors. Only variables with p-values <0.2 were included in the generalized linear models (GLMs). The correlation (correlation value >0.5) and/or multicollinearity (variance inflation factor >2–5) of independent variables was checked, and in that event, the variable which was considered to be less important and/or with the lower impact was not included. We performed binary logistic regressions using a stepwise method for removing variables that are not associated with the response variable one by one, while considering both the p-value of the variable and the Akaike Information Criterion. The models were tested for possible interactions. The best fitting model was selected by comparing the AIC weights for a set of fitted models. The final model was assessed in comparison with the null model using a likelihood ratio test. In addition, the individual association of the variables in the model was tested by Wald tests. In order to assess the predictive ability of the model, the area under the curve (AUC) was calculated using the "roc" R function. A p-value <0.05 was considered statistically significant.

Results

Documentation of vaccination

At the CH, the three doses of the pentavalent vaccine were verified in the vaccination card of all participants (100%) from Vientiane. Most of these children (65.5%) were vaccinated with

all three doses at the Children´s hospital, the others received some or all of the doses at another CH in Vientiane. In Bolikhamxay, the parents of 620 children (73.6%) presented the vaccination card. The vaccination entries in the hospital records were found for 83.3% of the children and for 56.8%, both sources were available.

Comparison of vaccination cards and hospital records

At the Children's hospital, it was possible to review the hospital records of all three doses for 88 (27.6%) participants, and for at least one dose for 155 (48.6%) participants. All vaccination dates matched between the vaccination card and hospital records. However, for 8 (2.5%) participants, the birth date entry in vaccination card and hospital records did not match.

In Bolikhamxay, both hospital records and vaccination cards were available from 479 (56.8%) participants. We discovered a number of discrepancies between the two documents. The birth dates of 34 (7.1%) participants differed between vaccination card or hospital records. From the 479 participants in Bolikhamxay with both hospital records and vaccination card, 180 (37.6%) had at least one discrepancy or mismatch between the hospital records & vaccination card date-pairs of at least one of the three doses. From these 180 participants, 43.3% had a mismatch in the dates for pentavalent 1 and 62.8% for pentavalent 3. 17.2% had mismatches for all three date-pairs. The date for pentavalent 3 in the hospital records was before the vaccination card in 71 (39.4%) cases while it was later in only 37 (20.6%) of the 180 participants. The proportion of mismatches increased from pentavalent 1 to 3. At the PH level, only 2.4% to 6.0% of the vaccination dates mismatched from pentavalent 1 to 3 (Table 2). At the DH and HC level, there was a higher proportion of mismatches between vaccination dates at the facility as compared to outreach. The highest proportion of mismatches (41.1%) was found at the health center level among the participants vaccinated directly at the facility. Here, also the largest mean discrepancy between the vaccination card and hospital records was also observed (-9.9 days) (S1 Table in S1 File).

Several other observations were made: In some cases (7.8%) among the 180 participants with both records, either pentavalent 2 or 3 or both, the hospital records had the same entry as pentavalent 1 and/or 2 in vaccination card; many of the mismatches differed by less than 7 days for at least 1 date-pair (23.9%); for 9.4% of participants all dates in the hospital records were before the vaccination card; for 7.2%, both the intervals were 4 weeks in the hospital records but not in the vaccination card and at least one of the dates in the hospital records was before the one in the vaccination card. On very few occasions, two doses were denoted with the same date or an obvious mistake was made with respect to the recorded year or the day. Because the vaccination card were less likely to be tampered with, the dates in the vaccination card were given priority in case of mismatches.

Table 2. Comparison between vaccination date pairs from the vaccination books and vaccination cards.

	Provincial hospital		District hospitals				Health center			
			Facility		Outreach		Facility		Outreach	
	n mismatch* /N	%	n mismatch /N	%	n mismatch /N	%	n mismatch /N	%	n mismatch /N	%
pentavalent 1	2/83	2.41	16/63	25.40	5/45	11.11	11/58	18.97	44/230	19.13
pentavalent 2	2/83	2.41	17/62	27.42	6/45	13.33	20/58	35.09	50/232	21.55
pentavalent 3	5/83	6.02	19/60	31.67	6/46	13.04	23/56	41.07	60/234	25.64

N = total number per group (in some instances, the dates were unreadable or missing and only the signature of the health care worker was present);

pentavalent = Diphtheria-Tetanus-Pertussis whole cell–Hepatitis B–*Haemophilus influenzae* Type B vaccine

* Mismatch = The date for the specific dose of the pentavalent vaccine in the hospital records and vaccination cards did not match

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Age at vaccination

The median age at vaccination with pentavalent dose 1, 2 and 3, and therefore also the difference between the median age and the recommended age, increased at the lower ranked health care facilities (Table 3) and with each dose, e.g. the median age at the first dose was 6.7 weeks at the central hospital level, but 9.4 weeks at the health center level when participants were vaccinated by outreach services.

The Kaplan-Meier curve (S1 Fig) shows that at the CH level, 50% of the children were vaccinated at the age of 6.7 weeks with pentavalent 1 compared to almost 9 weeks at the HC level. For pentavalent 3, there was a considerable delay at all lower ranked facilities. At the HC outreach level, 50% vaccination coverage was reached at 23.1 weeks (CI: 22.4–24.4) and considerably fewer children were vaccinated by 24 weeks (6 months) of age compared to CH, PH and DH levels (S1 Fig).

In order to estimate the timeliness of vaccination, the proportion of participants vaccinated at a given age was calculated (Fig 1). The proportion vaccinated at the recommended 6 weeks of age decreased from 67.7% at the CH to 32.4% at the HC, and outreach vaccination was as low as 12.7%. For pentavalent 2, most of the participants were still vaccinated within a week of delay at the CH and PH (55.5% to 55.8%, respectively). The majority of participants (ranging from 61.1% at the CH to 94.7% for DH outreach) were vaccinated with a delay of 2 or more weeks for pentavalent 3 at all locations.

Compared to the central hospital level, fewer participants who were vaccinated at the district and health center level received the third dose by 16 weeks (48% (95% CI [42.6–53.6]) at the central hospital level vs. 7.1% (95% CI [3.4–10.8]) at the district and 12.4% (95% CI [9.2–15.5] at the health center level) (S2 Fig). However, by week 24, more than 90% of the participants at the CH and PH level (94.7% (95% CI [92.2–97.1]) and 92.6% (95% CI [88.8–96.3]) respectively), as well as participants vaccinated directly at the DH level (91.5% (95% CI [86.5–96.6])) had received pentavalent 3. Fewer participants that were vaccinated at the HC level and during DH outreach services were vaccinated with the 3rd dose by 24 weeks of age (58.4% (95%CI [54–62.7], $p<0.0001$) (S2 Fig) when compared to the CH.

In order to investigate any association between delayed vaccination (defined as age at pentavalent 3 >16 weeks) and seroprotection against any of the five vaccine components, bivariate analyses were performed among those children vaccinated at the CH and PH level. Receiving the 3rd (last) dose of the pentavalent vaccination more than 16 weeks after birth was not associated with lower seroprotection (S2 Table in S1 File) in this study.

Table 3. Median age at vaccination in weeks according to health care level.

	Median (Interquartile range) age at vaccination[a]		
	pentavalent 1	pentavalent 2	pentavalent3
Health care level	**recommended at 6 weeks**	**recommended at 10 weeks**	**recommended at 14 weeks**
CH	6.71 (6.43–7.00)	11.43 (11.14–12.14)	16.14 (15.71–17.29)
PH	6.86 (6.71–7.43)	11.57 (11.14–12.71)	16.29 (15.71–17.89)
DH—facility	7.14 (6.71–7.71)	12.21 (11.71–13.57)	18.00 (16.86–19.96)
DH—outreach	8.5 (7.04–10.21)	13.57 (12.14–18.36)	20.14 (17.64–28.25)
HC—facility	7.14 (6.64–9.36)	13.14 (11.46–16.82)	19.71 (16.79–23.61)
HC—outreach	9.36 (7.29–12.14)	15.64 (12.68–20.86)	22.86 (19.00–29.71)

[a]The age was calculated with the date written in the YC, if the YC was not present, the date in the HR was used

Pentavalent = Diphtheria-Tetanus-Pertussis whole cell–Hepatitis B–Haemophilus influenzae Type B vaccine, CH = central hospitals, PH = Provincial hospital, DH = District hospitals, HC = Health centers

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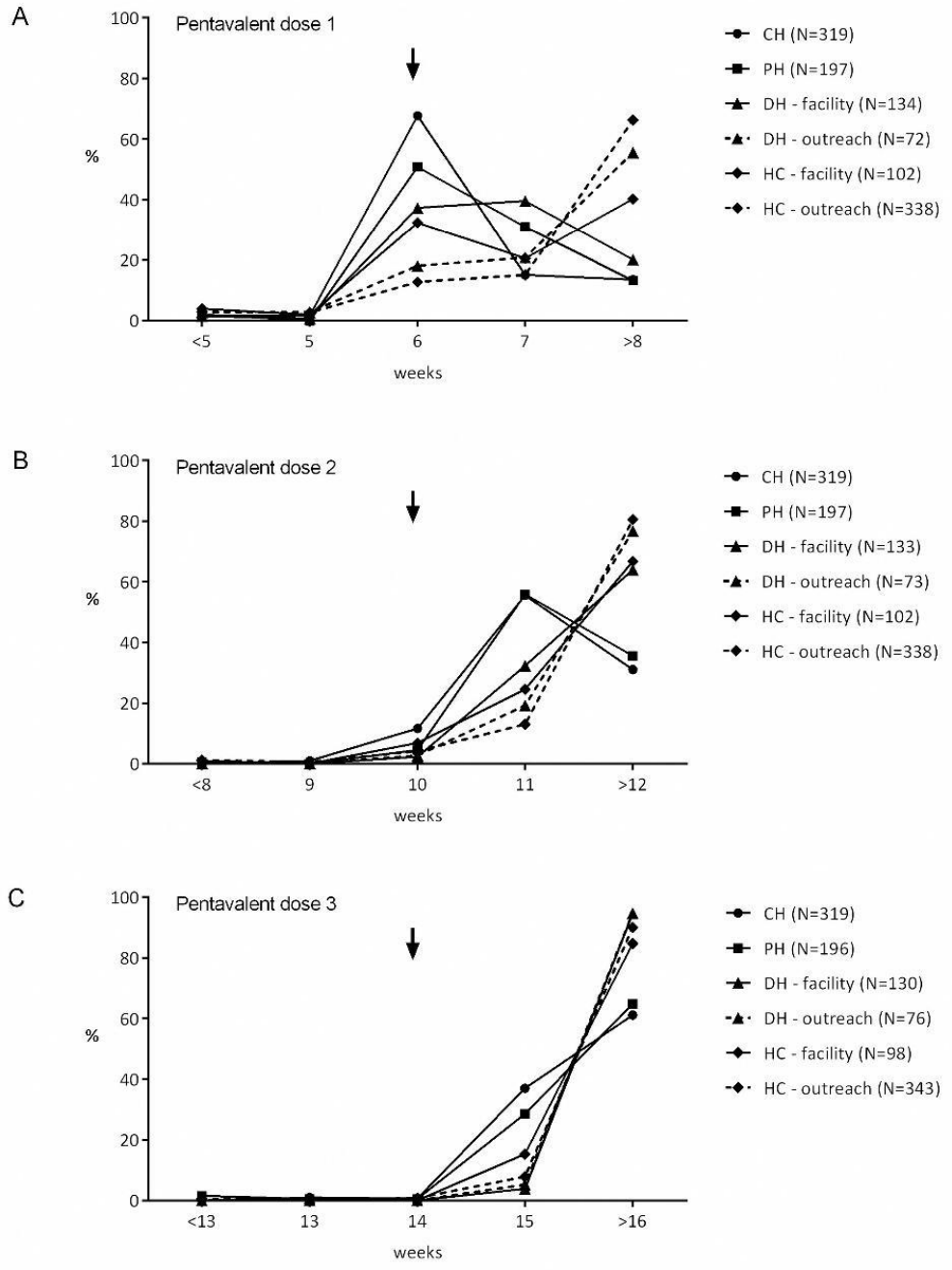
A
Pentavalent dose 1
%
weeks
<5
5
6
7
>8
CH (N=319)
PH (N=197)
DH - facility (N=134)
DH - outreach (N=72)
HC - facility (N=102)
HC - outreach (N=338)
B
Pentavalent dose 2
%
weeks
<8
9
10
11
>12
CH (N=319)
PH (N=197)
DH - facility (N=133)
DH - outreach (N=73)
HC - facility (N=102)
HC - outreach (N=338)
C
Pentavalent dose 3
%
weeks
<13
13
14
15
>16
CH (N=319)
PH (N=196)
DH - facility (N=130)
DH - outreach (N=76)
HC - facility (N=98)
HC - outreach (N=343)

Fig 1. Proportion of participants vaccinated with pentavalent 1, 2 and 3 at a given age by health care levels. Arrows indicate recommended week of vaccination. CH = central hospital level, DH = hospital level, HC = health center level. The age at vaccination was calculated based on the vaccination card, and in case the vaccination card was not available, the date in the hospital records was used.

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Interval between vaccination doses

At the CH level, the median interval between pentavalent dose 1 and 2 was 4.7 weeks (S3 Table in S1 File; S3 Fig) and only somewhat higher for outreach vaccination at the HC and DH level (5.1 and 5.4 weeks respectively). At the CH and PH level, 62–70% were vaccinated within the recommended interval of 4 weeks between dose 1 and 2 and dose 2 and 3. Considerably less participants were vaccinated within 4-week intervals in lower ranked facilities. Only very few participants were vaccinated within less than 4 weeks (rates ranging from 0.0% to 4.4%) at any health care level.

For most participants at the district and health center level, at least one of the two intervals (pentavalent 1 and 2 and/or pentavalent 2 and 3) was longer than 4 weeks (ranging from 57.1% to 83.2%) (S4 Table in S1 File).

Predictors for timely completion of vaccination with the pentavalent vaccine

We used timely completion with pentavalent 3 at 24 weeks as binary outcome and performed logistic regression analyses to identify predictors in Bolikhamxay province. The bivariate analyses (S5 Table in S1 File) showed that being an older mother with a higher education and a higher household income was more often associated with timely vaccination. Markers of inequity in access to health care (delivery at home, no ANC and no hepatitis B birth dose) all were also predictors of delayed vaccinations. Travel time to health care facilities, vaccination by outreach and not belonging to the Tai-Kadai ethnic group emerged as obstacles to timely vaccination.

In logistic regression analyses all variables associated with the outcome with a $p<0.2$ were included. However, since "place of birth" and "district" correlated with the "place of vaccination", these variables were excluded from analyses. "Travel time to the next health care facility" was used as surrogate for distance and "received ANC" was used as surrogate for ANC practices in the modelling. The variables "age of participant", "age of mother", "number of household members", "number of siblings" and "travel time to the next health care facility" were included in the logistic model as numeric variables. Several variables remained independently associated with the outcome (Table 4). Participants were more likely to have completed the primary vaccination series by the age of 24 weeks (6 months) if they were not vaccinated by

Table 4. Binomial generalized linear model showing the factors affecting completion of vaccination by 24 weeks.

Factors included in the final model	OR	95% CI	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	1.59	[0.53–4.74]	0.47	0.56	0.84	0.403
Mother's level of education (Secondary school/University; ref: none/primary education)	1.64	[1.09–2.47]	0.49	0.21	2.39	0.017
Number of siblings (numeric)	0.81	[0.69–0.94]	-0.21	0.08	-2.66	0.008
Age of mother (years) (numeric)	1.05	[1.01–1.09]	0.05	0.02	2.27	0.023
Hepatitis B birth dose (received; ref: not received)	2.14	[1.42–3.24]	0.76	0.21	3.62	<0.001
Place of vaccination (outreach service; ref: vaccinated at the HCF facility)	0.23	[0.14–0.36]	-1.48	0.24	-6.24	<0.001

OR = odds ratio; CI = confidence interval; std = standard; ref = reference; HCF = health care facility

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Table 5. Numbers of participants that received the hepatitis B birth dose according to their vaccination cards or vaccination records and the level of health care system.

	Health care level [a]						
	CH	PH	DH-facility	DH-outreach	HC- facility	HC-outreach	Total N
N received vaccinations at birth according to questionnaire	319	205	266	17	175	92	1074
n received birth dose according to written records	286	195	220	2	139	26	868
(%)	(89.7)	(95.1)	(82.7)	(11.8)	(79.4)	(28.3)	(80.8)

[a] Participants were grouped according to the place of vaccination with the vaccinations at birth. In the questionnaire, no distinction was made between BCG and hepatitis B birth dose. 88 participants were excluded because participants born outside study, dates not readable, missing, or only in month and year, or parents did not know place of vaccination

CH = central hospitals, PH = Provincial hospital, DH = District hospitals, HC = Health centers, N = total number per group

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outreach services and if they had received the hepatitis B birth dose. Furthermore, the probability of timely completion increased with age and education of mother but decreased by number of siblings. The fit of the overall model in comparison to the null model was assessed (p-value < 0.0001, AUC = 77.9%).

Hepatitis B birth dose and recall by health facility

At the CHs in Vientiane Capital, 89.7% had received the birth dose according to vaccination card or hospital records compared to 76% in Bolikhamxay (Table 5). All of the parents of participants from Vientiane Capital stated that their children had received the vaccination at birth (BCG and/or hepatitis B birth dose) at the Children's hospital or another CH. In Bolikhamxay at HC level, only 28.3% of children whose parents remembered that a vaccination was given at birth had record of the hepatitis B birth dose. These participants were almost exclusively born at home.

In Bolikhamxay, both hospital records and vaccination card were available from 479 participants and from those, 313 (65.3%) participants had a vaccination date for the birth dose. The vaccination dates matched in both hospital records and vaccination card for 91.7% of these participants.

The hepatitis B birth dose is recommended to be given within 24 h of birth and latest within the first 7 days after birth [39]. The vast majority of the participants in our study that received the hepatitis B birth dose, did receive it on the day of their birth (93.5%) and only 4.1% did receive it later but within 7 days after the birth date (S6 Table in S1 File).

Discussion

Reliable and centralized documentation is important for monitoring vaccination coverage and the quality of immunization programmes. In Bolikhamxay, when both hospital records and vaccination card were available, 38% of children had mismatches in at least one of the vaccination date-pairs, and for 63% of those, the date for pentavalent 3 did not match. At the PH level only 2.4% of mismatches were found for the first dose, but in lower ranked health facilities up to 19% of date pairs mismatched. The mismatches increased with each dose (up to 41% at the HC level). There were also 7.1% mismatches in birthdates between the two vaccination records. Mismatches in calendar days, months or years, are indications that dates are not entered simultaneously and at the same session in the vaccination card and hospital records. Vaccination books are usually assigned to each village and taken along to the village during outreach sessions. When the books or the vaccination card are forgotten, retrospective entry of dates

may be common. Vaccination dates may also be entered in the vaccination card in advance as a reminder for the parents, but then vaccination may have taken place at another date. Thus, the importance of written documentation needs to be emphasized at every level of the health system. In particular, the management of hospital vaccination books/records needs to be strengthened, the procedures of record keeping in both the vaccination card and the hospital records should be reviewed, and additional training of the health care staff is required to improve the reliability of entries.

Adherence to the vaccination schedule is considered an important quality aspect of routine immunization, even though there is no generally accepted definition of "timely vaccination" [15]. We observed a general delay in vaccination with the pentavalent vaccine, especially in health care settings below the PH level. The difference between median age at vaccination and recommended age at vaccination was highest for pentavalent 3. More than half the children vaccinated at the DH and HC level received all three doses later than recommended. The delay at the DH and HC outreach level may reflect challenges with scheduling outreach visits in those villages. If children miss one visit, they may have to wait for the next visit of the health care workers. In our previous study [20] in this cohort, the place of vaccination was an important predictor for seroprotection. The proportion of protected children was especially low in villages connected to health centers that were located more than one hour of travel time from their district hospital. These findings combined underline the need to strengthen vaccine management in the lower-ranked health care settings. In total, 22% of the children did not complete the immunization series with the pentavalent vaccine by the age of 24 weeks. While we did not find a negative impact of delayed vaccination on seroconversion rates at the CH and PH level, delayed vaccination increases the window of susceptibility and may facilitate disease outbreaks. Since the presence of maternal antibodies may interfere with the infant's humoral immune response after primary vaccination [40–42], the timing of the first vaccine dose is especially important. However, since the vast majority of the participants in this study were not vaccinated before the scheduled first dose of the pentavalent vaccine at 6 weeks of age, we could not investigate a possible impact of premature vaccination on antibody titers.

In bivariate analyses, essentially all indicators of poor access to health services were very specifically associated with delayed completion of vaccination with the pentavalent vaccine. Since this study included only children who completed vaccination, further studies could investigate whether these are also the risk factors for missing any of the doses of the pentavalent vaccine. After logistic regression analyses, lower education level of the mother, not receiving the hepatitis B birth dose as well as vaccination by outreach service, were independently associated with the delayed completion of full vaccination with the pentavalent vaccine. Due to the geography and infrastructure of the country, around 30% of the villages are classified as remote and are difficult to access. Outreach services should be conducted in at least four rounds per year; however, in 2016, it was estimated that only 9% of remote villages were visited four times [43]. In contrast to our findings, a study conducted in Sri Lanka found higher timeliness in rural areas compared to urban settings [5]. The education of the mother has also been shown in other studies [2, 4, 44] to be an important driver on timely immunization or timely completion of specific vaccinations. These results indicate that there is a need to further improve access to health services, especially in remote rural areas.

Almost all participants in Vientiane Capital but only two thirds of the participants living in Bolikhamxay had received the hepatitis B birth dose; however, this is probably a considerable overestimation of recipients in the general population, since we only enrolled participants with a full course of pentavalent vaccination. Recent data estimate the coverage for the birth dose in 2017 to be 55% nationwide [45]. Even among children who received all three doses of the pentavalent vaccine, only 28.3% had received the hepatitis B birth dose at the health center level in

areas covered by outreach. Although the overall number of home births in Lao PDR has decreased from 59% in 2010 [46] to 35% in 2017 [10], these findings are still concerning.

There are several limitations to this study. Those include that the specific place of vaccination (either outreach or on-site) was only available by parents' recall. Since only children with three doses were included, we cannot determine the number of children who missed one or two doses. Our questionnaire may also not have captured all risk factors for delayed vaccination and our findings may not necessarily be valid for the whole country although it shows a typical pattern.

Conclusions

During the past few years, major progress has been made in Lao PDR in vaccine coverage and seroconversions rates. In this study, we observed a general delay of vaccination with the pentavalent vaccine and discrepancies in vaccination records. Vaccination delay was associated with indicators of poor access to health services. To further improve the child vaccination programme, reasons for the discrepancies and inconsistency in vaccination documents should be investigated and training of health care staff in robust documentation and management of health records should be provided. We suggest to include timely completion of vaccination as a quality indicator for the national immunization programme in addition to coverage rates and seroconversion rates.

Supporting information

S1 Fig. Timeliness of each dose of the pentavalent vaccine according to the age and the health care levels. A. Timeliness of vaccination with pentavalent 1. B. Timeliness of vaccination with pentavalent 2. C. Timeliness of vaccination with pentavalent 3. Shaded areas indicate the 95% Confidence Interval. Graphs were truncated at 45 weeks to increase visibility. CH = vaccinated at central Hospitals in Vientiane, PH = vaccinated at provincial hospital, DH = vaccinated at district hospital level, HC = vaccinated at health center level. Dashed lines correspond to the median age of vaccination with the pentavalent vaccine. Participants for which the calculated age at vaccination was negative (date of dose before date of birth, indicating a mistake in documentation) were excluded from the graph.
(TIF)

S2 Fig. Proportion of participants vaccinated with the third dose of the pentavalent vaccine by health care levels. Mix = participants vaccinated at different health care facilities with one or two of the doses. CH = Central hospital level, DH = District hospital level, HC = health center level. Missing or unreadable dates were excluded from this figure. The age at vaccination was calculated based on the vaccination card, and in case the vaccination card was not available the date in the hospital record was used. The proportion vaccinated at PH, the DH and HC level was compared to the CH level. Data are presented with 95% CI. **** = $p<0.0001$.
(TIF)

S3 Fig. Difference of interval as recommended in schedule and calculated median interval between pentavalent dose 1 and 2 (A) and pentavalent dose 2 and 3 (B) in weeks according to health care level. The intervals were calculated based on the vaccination cards, and in case the vaccination card was not available the date in the hospital records was used. CH = Central hospital level, DH = District hospital level, HC = health center level. Missing or unreadable dates were excluded from this figure.
(TIF)

S1 File.
(DOCX)

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CHAPTER 7. Hepatitis B virus infection in the Lao People's Democratic Republic: A systematic review

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7.1. Contribution statement

The results presented in this literature review were gained from a collaborative work and the manuscript presented here is currently under preparation for submission to a peer-reviewed journal. Ms Hefele conducted the literature review and collated the HBV data. Ms Hefele and Dr Black compiled the first draft of the manuscript. All authors wrote and commented on the manuscript.

7.2. Bridging section

It is widely understood that hepatitis B represents a major public health problem in the Lao PDR, with 8 to 10% of the Lao adult population estimated to be chronically infected[4,43,44]. Hepatitis B vaccination was introduced in the Lao PDR around 2001 (although dates vary in literature) as part of the DTP vaccine. The hepatitis B birth dose was added to the national immunization schedule in 2003.

As a serious public health problem, hepatitis B has been a particular focus of the LaoLuxLab. The last research objective in this thesis was to evaluate the impact of hepatitis B vaccination in Lao adolescents. However; despite the obvious significance of hepatitis B in the Lao PDR, the understanding of the hepatitis B epidemiology is incomplete. At the beginning of this research work, a literature review was conducted, to summarize the existing literature on HBV prevalence and genotype data in the Lao PDR. The aim of the review was to provide a comprehensive overview over the data and identify knowledge gaps in order to inform vaccination policy.

It should be noted, that the study regarding the impact of hepatitis B vaccination (**CHAPTER 8**) was also included in the literature review since the manuscript was published before the review manuscript was finalized.

7.3. Abstract

Introduction

Even though hepatitis B is endemic in the Lao PDR, the understanding of the epidemiology of hepatitis B infection is incomplete. This article reviews the available literature about hepatitis B seroprevalence, risk factors and genotypes in the Lao population in order to provide an up-to date summary of the HBV epidemiology in the Lao PDR, identify knowledge gaps and provide public health recommendations.

Methods

Using PubMed/Medline and ScienceDirect, all studies reporting the prevalence of hepatitis B markers or genotype distribution in the Lao PDR published between 1990 and 2021 were systematically reviewed.

Results

The 21 studies included focused on the general population (including blood donors, women, and children) and risk groups (including health care workers and garment factory workers). The studies varied extensively in sample size, target population, methods, study location and time periods. The prevalence of the hepatitis B surface antigen (HBsAg) in blood donors was reported to be 8.7%-9.6% in 2003-2006. In the years 2011-2012, the reported HBsAg prevalence among women (including pregnant women) ranged from 0%-9.5% and among children aged 5-9 years from 1.7%-8.7%, depending on study location and age. The majority of strains characterized in the Lao PDR belonged to genotypes B and C.

Conclusion

Studies displayed considerable heterogeneity in populations, design and laboratory methods. A high HBsAg prevalence was reported in adults including pregnant women. Low infant vaccination coverage and compromised vaccine immunogenicity were found. Only two studies focused on HBV in risk populations, emphasizing the need for further studies to characterize hepatitis B epidemiology in potentially vulnerable groups. Hepatitis B infection continues to represent a substantial public health threat in the Lao PDR and needs to be monitored to inform health authorities and to counteract over-burdening of the health care system. In order to end mother to child transmission, vaccination coverage with the hepatitis B birth dose should be increased.

7.4. Manuscript

Introduction

Hepatitis B and C, responsible for 96% of all hepatitis mortality, cause more deaths than either HIV/AIDS, tuberculosis or malaria and yet seem to receive much less attention. The WHO African and Western Pacific Regions are the most affected by the Hepatitis B virus (HBV). Although most infections with HBV are asymptomatic, in some patients, acute infection can cause severe liver inflammation. Chronic infection with HBV can lead to liver cirrhosis and hepatocellular carcinoma [139,158]. The Coalition for Global hepatitis elimination estimated that globally 40% of liver cancer deaths could be attributed to HBV and the global number of HBV-related deaths was estimated at more than half a million in 2019 [16].

HBV is grouped into at least 9 recognized genotypes (A to I), and one presumable genotype “J”, each with a distinct geographic distribution. Evidence suggests that the HBV genotypes are related to HBV transmission modes and clinical outcomes [64,66,96,97]. In Asia, genotypes B and C dominate largely [63,111] and are associated with vertical transmission [96].

Even though HBV is recognized as a major public health threat in the Lao PDR, it is challenging to provide quantitative epidemiological data about the HBV prevalence in the country. This is due to several reasons, including an inadequate surveillance system [86], the geographic and ethnic heterogeneity of the country and limited HBV testing. Understanding the HBV epidemiology in the Lao PDR is important for improving vaccination, testing and treatment strategies.

Routine vaccination for children is free of charge in the Lao PDR. Since 2001, HBV containing vaccines were gradually introduced in the routine national immunization schedule at 6, 10 and 14 weeks of age. Currently, HBV vaccination is administered as part of the pentavalent routine infant vaccine in combination with diphtheria, tetanus, pertussis and *Haemophilus influenzae* type b (DTPw-HepB-Hib). Vaccination coverage rates vary regionally, but the nationwide coverage of 3 doses of the DTPw-HepB-Hib reached 84% in 2018 [157]. The monovalent HBV birth dose was introduced in the Lao PDR in 2003 [9,91], but coverage rates were only 55% in 2018 [157]. The low coverage rates for the birth dose are particularly concerning since mother-to-child transmission (MTCT) is one of the main routes of transmission in endemic countries [140]. To date, only pregnant women are routinely screened for HBV during antenatal care visits, but immunoglobulin and anti-viral treatment are not readily available in the Lao PDR. The high prevalence of HBV infections in the adult Lao population contributes to the high levels of liver cancer (22.4 per 100,000; 5th highest worldwide [33]) and will likely continue to perpetuate there.

Despite several studies in the Lao PDR, our insights in the epidemiology of Hepatitis B in the country are still fragmented. This systematic review, the first in the Lao PDR, will provide an up-to-date summary of HBV situation in the country. We aim to address the following questions: which data are available? Which conclusions can be drawn for national prevention and intervention strategies? What are the specific modalities of HBV transmission or susceptibility in particular risk groups? What are the prevalent HBV genotypes in the Lao PDR? What are current knowledge gaps?

Methods

Literature review and study selection

A systematic review of peer-reviewed literature on HBV in the Lao PDR was conducted following the PRISMA statement guidelines[73]. PubMed and ScienceDirect (Elsevier) were searched by using the following key words: “Lao PDR” (or “Laos” or “Lao People’s Democratic Republic”) in combination with “hepatitis B” (or “HBV”). The last day of search was 16.04.2021.

All articles were managed with the bibliographic management tool EndNote. Duplicate entries were removed. All peer-reviewed studies reporting prevalence of HBV infection or HBV genotype distribution in the Lao PDR and published between 01.01.1990 and 16.04.2021 were included. Reports

were screened and all reports which did not meet the eligibility criteria or were reviews, book chapters, case reports, or conference abstracts were removed (**Figure 14**).

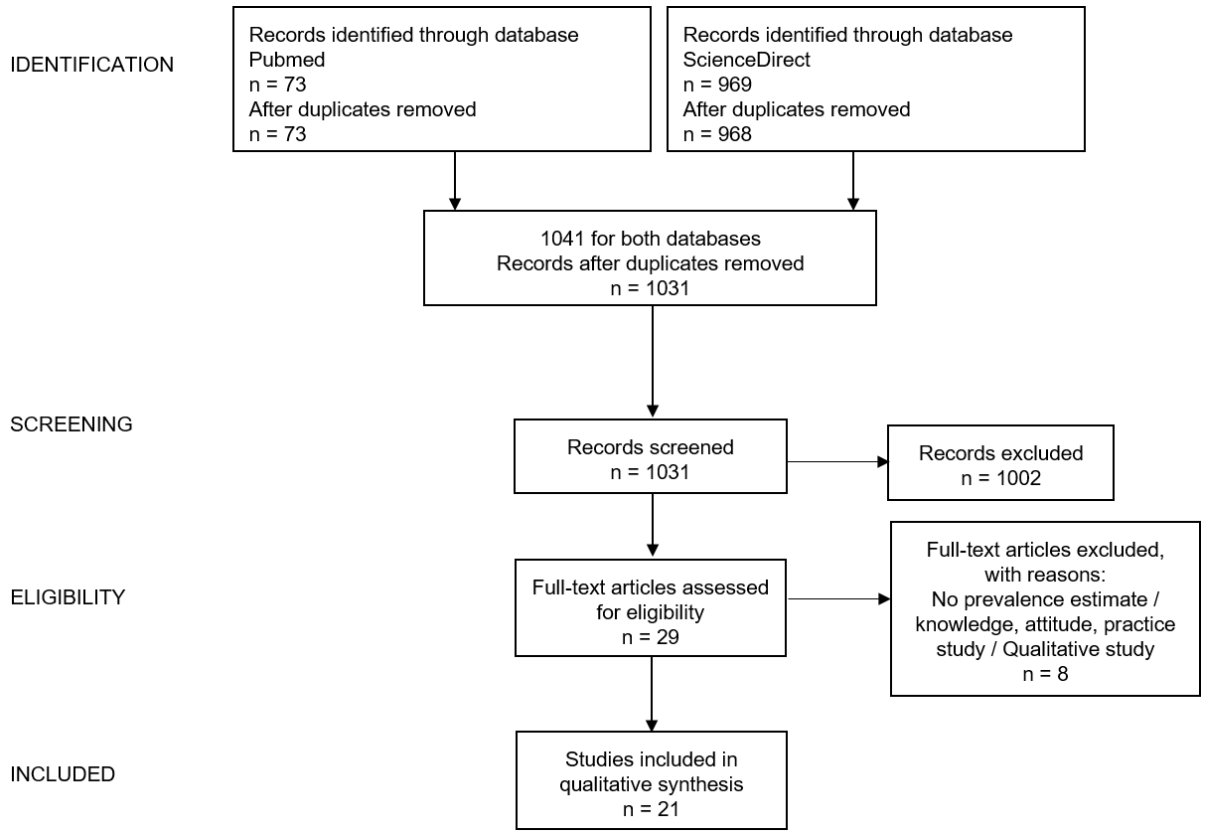

Figure 14 Flow diagram of the systematic review regarding Hepatitis B genotypes and Hepatitis B prevalence in the Lao PDR, 1990-2021

Data analysis

After selection of eligible studies, the following study data were extracted and entered into a table: study identification data (first author, publication year and journal of publication), study period, location, population, design, age of participants and estimates of prevalence of HBV markers or HBV genotype distribution. Prevalence of the hepatitis B surface antigen (HBsAg) was defined as the main primary outcome in addition to the prevalence of other HBV markers indicating past infection (anti-Hepatitis B core antigen antibodies; anti-HBc) or past infection/vaccination (anti-Hepatitis B surface antigen antibodies; anti-HBs). HBV genotype distribution was included as secondary outcome. Due to the scarcity of HBV prevalence studies in the Lao PDR and the heterogeneity of available studies, limitations and possible study biases were discussed for each study individually.

Results

General scope

Of the 1031 identified references, 1002 were excluded after screening the title or/and abstract. Of the remaining 29 articles, 21 were deemed eligible for inclusion in the systematic review (**Table 9**). From the three articles which investigated the genotype distribution of HBV in the Lao PDR [4,43,81], two studies also reported HBsAg prevalence [4,43]. The remaining 18 articles focused solely on the prevalence of HBV markers. Four studies were conducted in first-time blood donors [4,43,44,81], two studies investigated the aetiology of jaundice and hepatitis in Vientiane[11,113], 12 studies were conducted in the general population (including pregnant women) [9,14,162,165,28,40,41,45,51,58,78,161] and three studies focused on risk groups[10,68,163].

In terms of study design, almost all studies were cross-sectional (n=17), one study was a retrospective analysis of medical records, one study was an aetiological study, one study was a case-control study and one study was a prospective study.

Patients with acute jaundice

An early report investigated the aetiology of acute jaundice in three hospitals in Vientiane in 1995-1996 [11] (**Table 10**). In this study, HBV was identified as one of the major causes of acute viral hepatitis (10% IgM anti-HBc), the most common being Hepatitis A (14%). HBV was also identified as an important cause of hepatitis or jaundice in hospitalized patients in Vientiane from 2001 to 2004 [113].

HBV prevalence

All studies (including genotype studies) but one provided HBsAg estimates and 9 studies also investigated the prevalence of anti-HBs and anti-HBc antibodies. The 18 seroprevalence studies varied in sample size, target population, methods, location and time periods (**Table 10**, **Figure 15**).

Table 9 Overview over all articles included in the review

Year	Authors	Title
1998	Bounlu et al.	Acute Jaundice in Vientiane, Lao People's Democratic Republic
2007	Jutavijittum et al.	Seroprevalence of Hepatitis B and C virus infections among Lao blood donors
2008	Olinger et al.	Possible new hepatiits B virus genotype, Southeast Asia
2010	Syhavong et al.	The infective causes of hepatitis and jaundice amongst hospitalised patients in Vientiane, Laos
2012	Andernach et al.	A High Variability of Mixed Infections and Recent Recombinations of Hepatitis B Virus in Laos
2013	Jutavijittum et al.	Occult hepatitis B infections among blood donors in Lao PDR
2014	Black et al.	Hepatitis B virus in the Lao People's Democratic Republic: a cross sectional serosurvey in different cohorts
2014	Xeutavongsa et al.	Chronic Hepatitis B Prevalence among Children and Mothers: Results from a Nationwide, Population-Based Survey in Lao People's Democratic Republic
2015	Black et al.	Serosurveillance of Vaccine Preventable Diseases and Hepatitis C in Healthcare Workers from Lao PDR
2015	Komada et al.	Seroprevalence of chronic hepatitis B, as determined from dried blood spots, among children and their mothers in central Lao People's Democratic Republic: a multistage, stratified cluster sampling survey
2016	Jutavijittum et al.	High rate of Hepatitis B virus mother-to-child transmission in Lao People´s Democratic Republic
2016	Xaydalasouk et al.	Assessment of mother-to-child HBV transmission at the prenatal consultation in Vientiane, Laos
2016	Evdokimov et al.	Low and disparate seroprotection after pentavalent childhood vaccination in the Lao People's Democratic Republic: a cross-sectional study
2017	Choisy et al.	Prevalence of Hepatitis B Virus Infection among Pregnant Women Attending Antenatal Clinics in Vientiane, Laos, 2008–2014
2018	Xaydalasouk et al.	Seroprevalence and risk factors of hepatitis B and C virus infections in female workers of Lao garment factories
2019	Hefele et al.	Seroprotection at different levels of the health care system after routine vaccination with DTPw-HepB-Hib in Lao PDR
2019	Norizuki et al.	Serologic testing of randomly selected children after hepatitis B vaccination: a cross-sectional population-based study in Lao People's Democratic Republic.
2019	Latthaphasvan et al.	Perinatal hepatitis B virus transmission in Lao PDR: A prospective cohort study
2020	Hefele et al.	Lasting benefit of infant hepatitis B vaccination in adolescents in the Lao People's Democratic Republic
2020	Mangkara et al.	Hepatitis B virus in Lao dentists: A cross-sectional serological study
2021	Xaydalasouk et al.	Age-stratified seroprevalence of vaccine-preventable infectious disease in Saravan, Southern Lao People's Democratic Republic.

Table 10 Data regarding HBV prevalence in the Lao PDR

Study year	Ref	Method	Cohort	N	Study site	Age	N subset	HBsAg + (%)	IgM anti-HBc + (%)	anti-HBs + / anti-HBc - (%)	anti-HBs - / anti-HBc -(%)	anti-HBs + / anti-HBc + (%)	anti-HBs - / anti-HBc + (%)
1995-1996	[11]	ELISA	Patients with jaundice	208	VTN				10.0				
2001-2004	[113]	ELISA	Patients with jaundice	392	VTN			18.0	16.0				
2003-2005	[44]	particle agglutination test, ELISA	First-time blood donors	13897	VTN, BLX	17-66 y		8.70					
			Male				10099	9.7					
			Female				3798	6.2					
2004-2006	[4]	NA	First-time blood donors	9157	VTN, central provinces	NA		8.80					
2006	[43]	ELISA	First-time blood donors	906	VTN, BLX	16-53 y		9.60		1.7	54.6	26.0	17.7
2008-2014	[14]	ELISA	Pregnant women	13238	VTN	13-48 y		5.44					
2008-2009	[45]	RT/ELISA	Pregnant women	3000	VTN	15-49 y		5.80					
2011	[9]	ELISA	Infants	192	All	9 -16 m		0.50		59.90	28.10	5.70	6.30
					VTN	9-16 m	94	1.10		64.90	24.50	3.20	7.50
					LPB	9-16 m	98	0.00		55.10	31.60	8.20	5.10
			Pregnant women	388	All	16-46 y		8.20		7.50	43.00	29.90	19.60
					VTN	16-46 y	189	9.50		3.20	51.90	29.10	15.90
					LPB	16-46 y	199	7.00		11.60	34.70	30.70	23.10
			School children[1]	1689	All	5-19 y		7.90		17.60	61.90	9.10	11.40
						5-9 y	393	4.83		31.30	56.74	5.85	6.11
						10-19 y	1296	10.19		13.43	63.43	10.03	13.12
					LPB	5-19 y	596	8.40		22.00	53.40	14.40	10.20
						5-9 y	185	4.86		37.30	50.81	7.57	4.32
						10-19 y	411	14.11		15.09	54.50	17.52	12.90
					BLX	5-19 y	560	3.60		19.30	63.90	6.60	10.20
						5-9 y	185	4.32		28.11	61.62	4.32	5.95
						10-19 y	375	3.47		14.93	65.07	7.73	12.27

					SVK	5-19 y	533	11.80	10.90	69.20	5.80	14.10
						5-9 y	23	8.70	8.70	65.22	4.35	21.74
						10-19 y	510	11.96	10.98	69.41	5.69	13.92
2011	[51]	filter paper, CLIA	Children	911	All	5-9 y		2.09				
					VTN Capital	5-9 y	186	2.15				
					VTN Province	5-9 y	182	2.75				
					BLX	5-9 y	179	3.91				
					KMN	5-9 y	180	1.11				
					SVK	5-9 y	184	1.63				
			Mothers	911	All	15-45 y		4.10				
					VTN Capital	15-45 y	186	3.76				
					VTN Province	15-45 y	182	6.59				
					BLX	15-45 y	179	5.59				
					KMN	15-45 y	180	2.78				
					SVK	15-45 y	184	3.26				
2011	[78]	Filter paper, CLIA	Children	911	VTN, VTN province, BLX, KMN, SVK	5-9 y		2.30	0.7[2]			
			Mothers	911	VTN, VTN Province, BLX, KMN, SVK	15-45 y		4.40				
2012	[165]	Rapid test	Children	965	NA	5-9 y		1.70				
			Mothers	965	NA	15-45 y		2.90				
						15-19 y	4	0.00				
						20-24 y	85	3.53				
						25-29 y	294	2.72				
						30-34 y	275	3.27				
						35-39 y	176	1.70				
						40-45 y	131	3.05				
2013	[161]	ELISA	Pregnant women	200	VTN	14-39 y		8.00				
2013	[9]	ELISA	Pre-school children	132	HPN	1-4 y		4.50	13.60	65.10	11.40	9.90

2013	[10]	ELISA	Health care workers	1128	VTN, BLX, HPN	15-69 y		8.00	21.00	30.20	32.20	16.70
2013-2014	[28]	ELISA	Vaccinated children	1039	VTN, BLX, KMN	9-50 m		0.96	34.1	58.13	3.85	3.95
			Mothers	1105	VTN, BLX, KMN	15-50 y		7.00	3.71	56.02	21.45	18.82
2015	[163]	ELISA	Garment workers	400	VTN	15-57 y		4.01	3.26	50.13	25.81	20.8
2015	[58]	RT, ELISA, PCR	HBV-infected mothers	153	VTN	24-30 y		100				
			infants	120	VTN			4.2				
2017	[40]	ELISA	Vaccinated children	805	BLX	8-28 m		0.75	69.94	26.96	1.86	1.24
2017	[162]	ELISA	General population	2463	SAR	5-90 y		3.8	9.9	56.9	15.8	17.4
						5-10y	258	0.00	39.53	58.91	0.78	0.78
						11-20y	639	2.80	11.42	73.08	8.45	7.04
						21-30 y	475	4.60	4.63	65.26	16.21	13.89
						31-40 y	336	5.40	4.46	54.17	19.35	22.02
						41-50 y	313	4.50	3.83	46.33	18.85	30.99
						>50 y	442	5.00	4.52	33.03	29.64	32.81
2018	[41]	ELISA	Adolescents	779	All	11-18 y		2.96	17.35	73.14	5.14	4.37
					VTN	11-18 y	388	1.55	19.33	74.23	3.87	2.58
					BLX	11-18 y	391	4.10	15.38	72.05	6.41	6.15
2018	[68]	ELISA	Dentists	317	VTN	15-63 y		5.0	13.6	48.6	19.6	18.3
			Male				104	7.0	13.5	37.5	20.2	28.8
			Female				213	3.8	13.6	54	19.2	13.1

VTN = Vientiane, BLX = Bolikhamxay, KMN = Khammouane, HPN = Huaphan, LPB = Luang Prabang, SVK = Savannakhet, SAR = Saravan; m = months, y = years, NA = not available, CLIA = chemiluminescence immunoassay

[1] Data in study [9] is reported only for the overall age group. The data was requested from the author and split into the age groups 5 to 9 and 10 to 19 for the purpose of this review.

[2]anti-HBs IgG overall (no information about anti-HBc status given)

General population

The first estimate of HBV infection in the adult population was based on 3 studies in first-time blood donors from 2004 to 2006 [4,43,44]. The HBsAg prevalence ranged from 8.7%-9.6% (**Table 10**, **Figure 15**). In two of those studies, HBsAg prevalence decreased with age and men were significantly more frequently affected than women [43,44]. The study conducted in 2006 reported serological evidence of past infection in 43.7% of the study participants [43].

Reported HBsAg prevalence estimates for women ranged from 0%-9.5% in the general population (**Table 10**, **Figure 15**). The earliest estimate of 6.2% chronic HBV infections in women was from the above study focusing on blood donors between 2003 and 2005[44]. Similarly, a hospital-based study in Vientiane from 2008-2009 found 5.8% of pregnant women aged 15-49 years to be HBsAg positive[45] (**Table 10**, **Figure 15**).

Between 2011 and 2012, four other studies characterized the HBV prevalence in women and children[9,51,78,165].

In 2011, a stratified, multistage, cluster sampling survey was conducted in central Lao PDR including 911 mother-child pairs[51]. The overall HBsAg seroprevalence was 4.1% in the mothers (15-45 years) (**Table 10**, **Figure 15**). Estimated HBsAg prevalence rates differed between study sites in Vientiane municipality (3.8%), Vientiane province (6.6%), Bolikhamxay (5.6%), Khammuane (2.8%) and Savannakhet (3.3%).

In a similar study in mothers (15-45 years) and their children in the same central provinces in the same year (2011)[78], found an HBsAg prevalence of 4.4% in the mothers.

A non-randomized, cross-sectional seroprevalence study including multiple cohorts[9] reported 9.5% and 7% HBsAg rates in pregnant women (16-46 years) in Vientiane and Luang Prabang, respectively, and nearly half of all pregnant women were positive for anti-HBc (49.5%).

In 2012, in another multistage, cluster sampling mother-child survey, HBsAg prevalence was only 2.9% in mothers (15-45 years)[165], but there was no indication of the specific study sites within Lao PDR.

Tree of the mother-child studies[51,78,165] used cluster sampling to estimate HBsAg prevalence rates in the Lao PDR, but they differed in their testing methodology. In two studies[51,78], samples were collected onto filter paper via finger prick and analysed by chemiluminescence immunoassay while in another study[165], a rapid test was used to determine HBsAg status. The authors of one study [51] remarked in their Discussion on issues regarding the utilization of dried blood spots in HBV serosurveys since there are not many reports investigating the diagnostic reliability in the context of field work.

A non-randomized cross-sectional seroprevalence study conducted in 2013/14 in Khammouane, Vientiane and Boulhikhamxay provinces[28] also recruited mother-child pairs, but focused on children

with a complete course of the DTPw-HepB-Hib vaccine. Overall HBsAg prevalence in mothers was 7%, higher than the overall HBsAg prevalence reported in mothers by some of the above studies[51,78,165], indicating regional variation or possibly different sensitivity and specificity of the techniques used for assessing HBsAg positivity.

A study conducted in 2013 in Vientiane capital reported the HBsAg seroprevalence to be 8% in pregnant women (14-39 years)[161]. Another retrospective study during 2008-2014 described HBsAg prevalence among pregnant women visiting the Mahosot hospital in Vientiane as 5.4% [14]. A slight yet steady decline of the prevalence was reported over the period of the study. These rates in pregnant women were somewhat higher than the prevalence of HBsAg reported in women in two of the mother-child studies[51,165].

The cross-sectional study conducted in Saravan reported 33.2% of the total tested population to have anti-HBc antibodies indicating previous exposure to HBV. The proportion of exposed individuals increased with age and the proportion of exposure in males was significantly higher than in females (37.4% and 30.0%; $p<0.0001$). Overall, 5% of the general population were chronically infected[162].

Children and adolescents

MTCT is one of the main routes of transmission in endemic countries[140]. The hospital-based study in Vientiane in 2008/09[45] which found 5.8% pregnant women aged 15-49 years to be HBsAg positive reported that as many as 21% of infants born to these HBsAg positive mothers were also HBsAg positive (**Table 10**). Unfortunately, the vaccination history of the children was not recorded in this study and it is unknown if they had received the HBV birth dose, which was introduced in 2003.

MTCT was investigated in a prospective-cohort study on HBsAg positive pregnant women and their new-borns from 2015-2017 in Vientiane Capital[58]. HBV vaccination was provided for all new-borns according to the immunization schedule. The authors reported a relatively low rate of MTCT considering that Hepatitis B immunoglobulin and anti-viral treatment are not readily available in the Lao PDR: 4% of children born to chronically infected mothers were positive for HBsAg. 15 of the 120 infants showed unsatisfactory protective antibody levels after vaccination when tested at 6 months of age (anti-HBs <10 IU/L), indicating the need for improving vaccine management.

The three multistage cluster sampling studies[51,78,165] found an overall HBsAg seroprevalence between 1.7% and 2.3% among 5 to 9 year old children (**Table 10**, **Figure 15**). Again, the HBsAg prevalence reported by another study[9] was more than twice as high (4.8%) in children of the same age range.

In a study from 2011[78], participants were tested for both HBsAg and anti-HBs. The study reported that only 0.7% of all the included children had a positive anti-HBs titer (with or without documented

vaccination) approximately 3-9 years after being presumably vaccinated. None of the children with a documented vaccination history showed protective anti-HBs titers (here defined as 10 UI/L).

A separate study[9] reported substantial heterogeneity in terms of vaccination coverage and health care access in the Lao PDR in 2011. Four sub-populations from different age groups were investigated regarding the prevalence of HBV markers. While infants showed a low prevalence of HBsAg (0.5%) and a relatively high rate of the serological vaccination profile (59.9%), school children born before and after the introduction of the vaccine in 2001 had a much higher HBsAg prevalence (7.9%). In 2011, school children (age 5-9 years) were included in this study[9] and one mother-child study[51] from the same location. While the prevalence of HBsAg in these school children reported from Bolikhamxay was similar in both studies (3.6% and 3.9%), the mother-child study[51] reported only 1.63% of the children to be HBsAg positive in Savannakhet, in contrast to the school children study[9], which found 8.7% of the children to be infected. Reasons for the discrepancy in HBsAg prevalences are not clear, but the studies differed greatly in design and methodology. In the mother-child study[51], participants were selected randomly, samples were collected onto filter paper via finger prick and analysed by chemiluminescence immunoassay while in the school children study[9], recruitment took place in the framework of a national measles and rubella vaccination campaign, samples were taken by venipuncture and analysed by enzyme-linked immunosorbent assay.

Although HBV vaccination was introduced in the national immunization schedule in 2001, only 13.6% of pre-school children (1-4 years) from a rural area in Huaphan province in Northern Lao PDR showed the serological vaccination profile in 2013 and 4.5% were positive for HBsAg. This indicated that vaccine coverage was very low in this setting even 10 years after the introduction of the vaccine[9] (**Table 10**, **Figure 15**).

In a study in 2013/14 less than 1% of children aged 9-50 months with documented three doses of DTPw-HepB-Hib were chronically infected, despite a low proportion of children showing a serological vaccination profile (34%)[28]. These results show that in addition to low vaccine coverage, vaccine immunogenicity is a problem in the Lao PDR. A follow-up study in 2017 reported a considerable improvement in vaccine immunogenicity and a decrease in HBV infection rates: HBsAg prevalence declined from 1.8% to 0.8%, when only children in the same age range were compared between 2013/14 and 2017[40].

In 2018, a cross-sectional seroprevalence study investigated the impact of the HBV vaccination in central Lao PDR in randomized adolescent school children from 11-18 years, born just before or after the infant vaccine introduction[41]. In this study, the proportion of students with a serological vaccination profile increased after the introduction of HBV vaccine and a sizable number were still protected 11 years after vaccination. In addition, the prevalence of past infection decreased.

Risk groups

Health care workers

A study conducted in 2013 revealed a very high proportion of health care workers to be susceptible to HBV [10]. Most participants included in the study were female (79.8%). 8% were HBsAg carriers (11.5% in males and 7.1% in females) and about half (48.8%) had anti-HBc antibodies indicating previous exposure (**Table 10**). The HBsAg prevalence of 7.1% in female health care workers was higher than reported for women in some studies[51,78,165], but is comparable to others[9,28,161]. Dental workers in a study from 2018 also showed a high proportion of previous exposure (37.8% anti-HBc+) and 5% were chronically infected[68] (**Table 10**).

Garment factory workers

A study in 2014 focused on female garment factory workers aged 15-57 years as a vulnerable population[163]. Although the HBsAg prevalence of 4% was lower than expected (**Table 10**), the data suggested a significant association with sexual risk behaviour and very low levels of knowledge and awareness regarding HBV. More than 40% of the women showed evidence of past infection with HBV.

Risk factors or predictors associated with HBV infection

Although some studies found no risk factors associated with HBV infection status[83], others have identified significant associations. HBsAg prevalence among blood donors was higher in males and increased significantly with age[43,44]. Chronic infection in the mothers was a risk factor for chronic infection in children aged 5-9 years in two studies [51,165]. Being born outside a health care facility was significantly associated with chronic HBV infection in one of the studies[51], but not in the other. One study reported decreasing HBsAg positivity rates in pregnant women according to study year, but not age [14]. Regarding the health care workers, HBsAg prevalence was higher in older participants and considerably lower in central hospitals, indicating that there may be differences in awareness and/or safety policies at different levels of the health care system[10].

Genotype distribution

We identified only 3 studies with HBV genotyping data in the Lao PDR (**Table 11**). The earliest study, published in 2008, reported a new genotype I [81]. In another study, phylogenetic analyses of 42 HBV strains from HBsAg positive first time blood donors revealed mixed infections of sub-genotypes B1, B2, B4, C1, C5, I1 and I2 [4]. The third study from 2014 reported strains belonging to genotype B, C and I [43]. Besides B, C and I, no other genotypes were reported for the Lao PDR. In all three studies, genotypes B and C, or a mix of both, were found most often.

Table 11 Summary of the studies conducted on prevalence of HBV genotypes in the Lao PDR

Ref	Study location	Year of sample collection	study population	n	Genotypes			
					B	C	I	Mixed (B, C, I)
[81]	VTN Capital, central provinces	NA	blood donors	386	163 (42.2%)	204 (55.4%)	19 (4.9%)	
[4]	VTN Capital	2004-2005	blood donors	42	14 (33.3%)	1 (2.4%)	1 (2.4%)	26 (61.9%)
[43]	VTN Capital, VTN Province, BLX	2006	blood donors	43	19 (44.2%)	21 (48.8%)	3 (6.97%)	

VTN = Vientiane; BLX = Bolikhamxay Province; NA = not available; n = samples screened

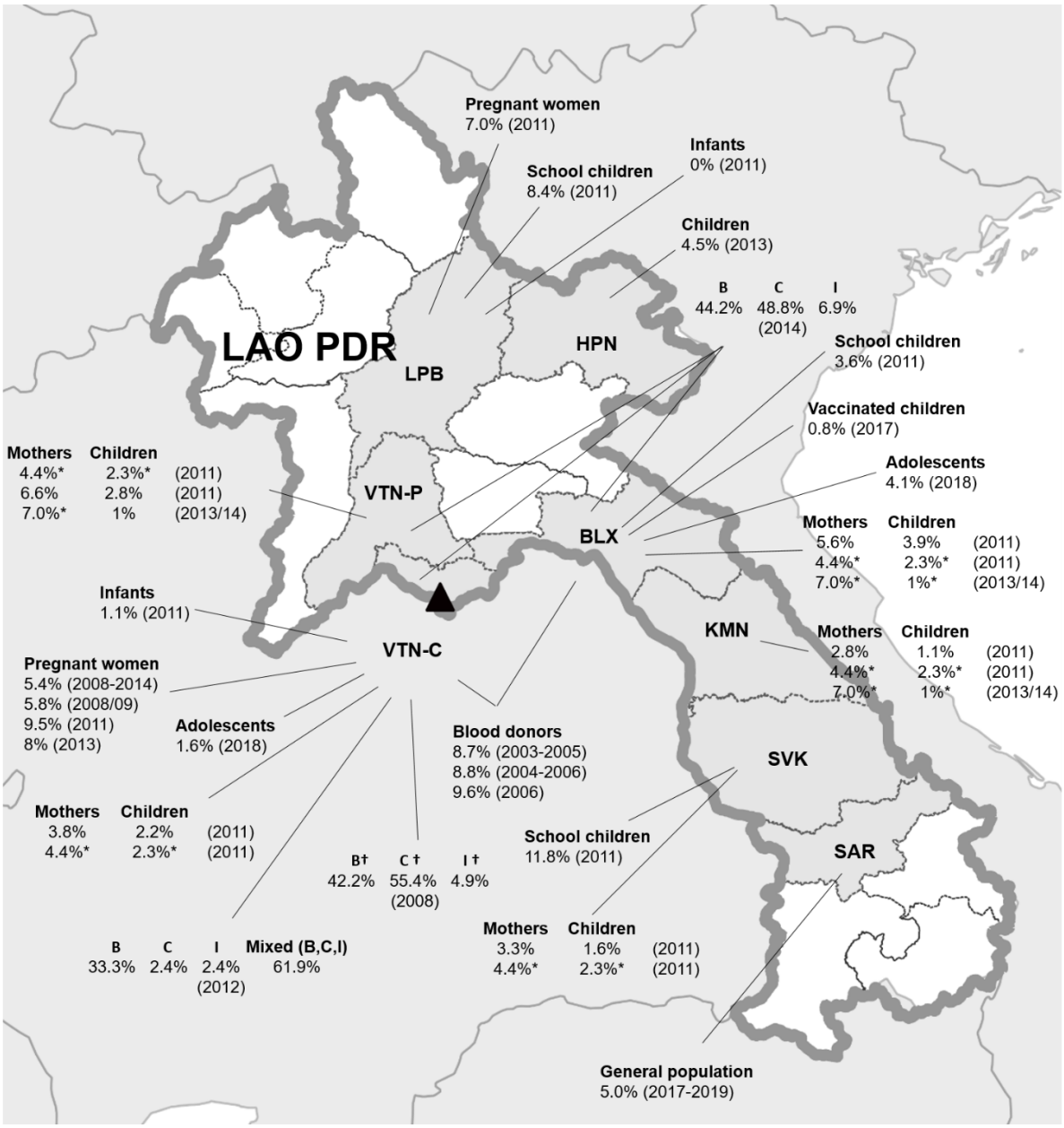

Figure 15 Geographical distribution of HBsAg prevalence in the general population and HBV genotypes in the Lao PDR. HBsAg prevalences in sub-populations are depicted per province (if information was given in the article), with the study year in brackets. * = HBsAg estimate was given as an overall estimate of several study locations. † = study location in article stated as Vientiane Capital and Central provinces. The location of Vientiane Capital is indicated by the black triangle. LPB = Luang Prabang, HPN = Huaphan, VTN-P = Vientiane Province, VTN-C = Vientiane Capital, BLX = Bolikhamxay, KMN = Khammouane, SVK = Savannakhet, SAR = Saravan.

Conclusion & Outlook

This literature review provides an overview of studies investigating the genotype distribution and HBV serology in different subgroups and regions and highlights that our understanding of the epidemiology of Hepatitis B in the Lao PDR is incomplete. The aim of most of the studies was to estimate the rates of

chronic infection in the Lao population; however, there are also some studies investigating the exposure to HBV, the level of protection conferred through vaccination against HBV or the genotype distribution.

Prevalence studies varied in study design and laboratory methods and included different population groups and therefore generalised statements about hepatitis epidemiology in the Lao PDR should be interpreted with caution. However, several important conclusions can be made, namely; high rates of chronic infection in adults, including women of child-bearing age; low infant vaccination coverage and compromised vaccine immunogenicity in particular regions; recent evidence of reduction in infection in adolescents born after vaccine introduction.

The earliest estimates of chronic HBV infection in the Lao PDR ranging from 8.7-9.6% were based on studies in blood donor samples derived from Vientiane Capital and central provinces in the years 2003-2006[4,43,44]. The classification of countries into high (≥8% HBsAg prevalence), intermediate (2-7%) and low (<2%) HBV endemicity is internationally recognized[105]. Based on this, Lao PDR would be considered as "highly endemic". However, while blood donors may represent an approximation for the general population, these studies were only conducted in central Lao PDR and do not provide any further information about other characteristics of the participants, such as for example ethnicity. In addition, the data may not mirror the current situation anymore as they are more than 14 years old.

Subsequent serological surveys provided further HBsAg estimates for women (ranging from 0-9.5%) and children and adolescents aged 8 months to 19 years (ranging from 0%-11.8%) [9,14,28,40,41,44,45,78,161,165]. One study estimated the rate of past infection for women at 40.3%[28]. Drawbacks of these studies include non-randomized study design and the utilization of different testing methodology, which makes it difficult to compare the results. In addition, studies solely including women may underestimate the burden of hepatitis B. HBV screening of pregnant women and raising awareness in the general population are still very important public health interventions which need to be strengthened. Since MTCT is assumed to be the main route of transmission in the Lao PDR, it is unsurprising that maternal infection was identified as risk factor in two studies[51,165]. Therefore, it is crucial to increase the coverage of the hepatitis B birth dose in order to reduce the transmission of hepatitis B in the Lao PDR. High infection rates represent a major public health challenge as treatment options in the country are still limited. Only 18% of patients in a retrospective cohort study conducted in 2018 describing the treatment of HBV infections in Vientiane Capital were reported to receive antiviral treatment[86].

Vaccination efforts will likely reduce the prevalence of chronic HBV in the Lao PDR in the long run, even though it is difficult to produce generalizable data on vaccine coverage and vaccine-induced protection due to the geographic and ethnic heterogeneity (which may play an important role) in the country[54]. The aim of the World Health Organization - Western Pacific Region was to reduce HBsAg-prevalence to less than 1% in children aged 5 years and increase national coverage with both timely

Hepatitis B birth dose (given within 24 hours of birth) and the third Hepatitis B dose to ≥95% by 2017[134]. At the moment, national coverage rates are at 55% and 85% for the birth dose and the third Hepatitis B dose, respectively[156]. It is unlikely that the target of reducing the HBsAg prevalence under 1% in children below 5 years is met in the Lao PDR in the near future. To date, most of the data regarding the impact of vaccination are derived from studies that did not randomly select participants or only report the prevalence of HBsAg which hampers the possibility to extrapolate the data or to assess the levels of vaccine-induced protection. One cross-sectional randomized study reported decreasing exposure and infection rates after the introduction of HBV vaccination[41].

Despite the overall high prevalence of HBV infection in the Lao PDR, there are only three studies focusing on risk groups. In 2013, a high proportion of Lao health care staff was found to be susceptible to HBV and/or under-vaccinated[10]. In addition, nearly half of the health care staff showed evidence of past exposure to the virus (48.9%). In the same year, a cross-sectional survey among students of health care professions was carried out in Vientiane Capital regarding their vaccination status, knowledge and awareness[89]. Less than one third (21%) were fully vaccinated against HBV. When asked about the reason for not being vaccinated, the most common answer was that they did not know where to get vaccinated. At present, there is no national policy for the immunization or serological screening of health care workers, except for influenza vaccination. A clear occupational health vaccination policy is needed to protect health care workers and their patients and other at-risk populations. Furthermore, campaigns promoting vaccination and raising awareness of the risks for health care providers and their patients may prove beneficial.

HBV data from vulnerable populations are needed; for example from sex workers and men who have sex with men. Furthermore, thalassemic and haemophilic patients and possible HBV co-infections with dengue, malaria or HIV are not yet investigated. The ethnic diversity in the Lao PDR poses another obstacle for the characterization of the HBV burden in the country. HBV infection rates among ethnic groups are not well described, apart from one study which took place in Saravan province[162] and could depend on local customs and risk practices such as tattooing, piercings, birth practices, sexual exposure etc.

The majority of the HBV strains characterized in the Lao PDR belonged to genotypes B and C. The distribution of genotypes found may have implications for the public health responses in the country, as genotype C was reported to show higher rates of progression from cirrhosis to hepatocellular carcinoma as compared to genotype B or other genotypes and showed a lower response rate to interferon[96,97].

In conclusion, Hepatitis B is still an important public health problem in the Lao PDR and more research is needed to better characterize its epidemiology. Interventions including HBV vaccination face many challenges but have showed first successes, at least in populations in central Lao PDR. In order to reduce the huge burden of HBV and its related morbidity and mortality in Lao PDR, such control measures

need to be strengthened and sustained for the foreseeable future. Furthermore, the management of the existing HBV burden needs to be improved through increased testing and treatment capacities.

Limitations

As a systematic review was performed, we did not include any existing grey literature on the topic. Study identification and data extraction were performed by one investigator only, possibly leading to selection bias. In addition, we cannot exclude the possibility that relevant articles were missed because they are not indexed in the databases used for the literature search. The heterogeneity of the studies concerning design, locations, methods and target population hampered the comparability of the findings.

CHAPTER 8. Lasting benefit of infant hepatitis B vaccination in adolescents in the Lao People's Democratic Republic

8.1. Contribution statement

The study presented in this section was published as:

Lisa Hefele, Souphaphone Vannachone, Vilaysone Khounvisith, Phonethipsavanh Nouanthong, Somphou Sayasone, Sengchang Kounnavong, Phetsavanh Chanthavilay, Claude P. Muller, Antony P. Black, *Lasting benefit of infant hepatitis B vaccination in adolescents in the Lao People's Democratic Republic*, International Journal of Infectious Diseases, Volume 93, 2020, Pages 217-223, ISSN 1201-9712, https://doi.org/10.1016/j.ijid.2020.01.055.

Ms Hefele was involved in the conceptualization of the study from the beginning. She was responsible for data curation, the formal analysis, the writing of the initial draft and for reviewing and editing of the finalized version. An author contribution statement signed by all authors was supplied separately. The supplement material can be found in Appendix **C.f**.

8.2. Bridging section

Serology data may help in the assessment of vaccination programmes and the progress made towards disease reduction or even elimination. By analysing the serological HBV profiles, it is possible to distinguish between vaccination and natural exposure to the virus, which facilitates the interpretation of the study findings. Hepatitis B vaccination was introduced in the Lao PDR around 2001 [9]. Most research studies in the country focused on adults, born before vaccine introduction, or children born several years after the introduction. Data regarding the birth cohorts born just before and right after the implementation of Hepatitis B vaccination was lacking.

The last research article presented in this thesis focused on the prevalence of HBV serological markers in adolescents born before and after the introduction of HBV vaccination in the Lao PDR to assess its impact.

8.3. Publication

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Lasting benefit of infant hepatitis B vaccination in adolescents in the Lao People's Democratic Republic

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ABSTRACT

Objectives: Hepatitis B is endemic in Lao PDR with 8–10% of the adult population being chronically infected. We investigated the impact of hepatitis B vaccination on infection in adolescents born shortly before and after the introduction of the vaccine in 2001.
Methods: 779 students from Vientiane Capital and Bolikhamxay province were tested for HBV markers by ELISA. Socio-demographic information was collected with a standardized questionnaire. Predictors/risk factors for seroprotection or exposure to hepatitis B infection were assessed by bivariate and multivariable analyses.
Results: The prevalence of a serological vaccination profile increased significantly after the introduction of the vaccine (13.2%–21.9%, $p < 0.05$, in Vientiane; 3.0%–19.7%, $p < 0.001$, in Bolikhamxay), which translated into at least a 2-times lower prevalence of past infection. In logistic regression, older students in Bolikhamxay were less likely to be vaccinated and more likely to have been infected by HBV in the past.
Conclusion: Even though this study documented a sizable and lasting reduction in past hepatitis B infections in adolescents born after the introduction of infant hepatitis B vaccination, the overall levels of protective anti-HBs were low and warrant at least the introduction of a booster for adolescents. Furthermore, we suggest improving the coverage of the hepatitis B birth dose.

Introduction

Hepatitis B is a major public health challenge with the highest disease burden in the WHO Western Pacific Region and the WHO African Region (>6% chronic carriers). Since the 1980s, vaccines against hepatitis B virus (HBV) have been available to protect against infection, chronic disease and liver cancer (World Health Organization, 2020a). In Lao PDR, hepatitis B is endemic with chronic infections in 8–10% of the adult population (Black et al., 2014). The introduction of HBV vaccines into the national immunization schedule in Lao PDR started in 2001, with three doses at 6, 10 and 14 weeks, followed by the introduction of the HBV birth dose in 2003, although dates vary according to sources (Black et al., 2014; Phoummalaysith et al., 2018; Xeuatvongsa et al., 2014; Kolwaite et al., 2016). Currently, the three HBV vaccination doses are part of a pentavalent vaccine, in combination with diphtheria, tetanus, pertussis and *Haemophilus influenzae* b (DTPw-HepB-Hib) (Black et al., 2014; Phoummalaysith et al., 2018). The majority of HBV infections occur perinatally and during early childhood, when the risk of chronic infection is highest. Therefore a high coverage with the birth dose (monovalent) is crucial to prevent chronic infection (World Health Organization, 2020a). There is little information about the uptake of the birth dose during the years early after its introduction but even in 2017, the nationwide coverage was only 55%. The percentage of children with 3 doses of the DTP-HepB vaccine (DTPw-HepB-Hib since 2009) increased gradually from 57% in 2006 to 89% in 2014 and 85% in 2017 (WHO, UNICEF, 2018).

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Besides coverage, vaccine response was as low as 37.9% in a study in 2013/14 (Evdokimov et al., 2017). However, recent improvements of the immunization programme and vaccine management resulted in a dramatic increase in vaccine response and typical protection levels against infection and chronic disease in small children (Evdokimov et al., 2017; Hefele et al., 2019).

In 2011, we reported that 17% of school students (age 5–19) from three different provinces had a past infection and 3.6% were chronically infected. Less than 20% had antibodies against the hepatitis B surface antigen (anti-HBs) in absence of antibodies against the hepatitis B core antigen (anti-HBc), indicating vaccination (Black et al., 2014).

In the current study, 17 years after the introduction of HBV vaccination, we assessed the long-term benefit of HBV vaccination for adolescents. Adolescents aged 11–18 years in 2018 were born at the time of and after the introduction of the HBV vaccination in Lao PDR. We aimed to investigate the impact of vaccination on protection rates and persistence of infections before they enter into the next phase in their lives with new risks of HBV exposure.

Methods

The study was approved by the Lao National Ethics Committee (Reference number 022/NECHR) and the Institutional Review Board of the Institut Pasteur (Reference number 9).

Participants

Between March and June 2018, 4 and 5 schools were selected in Vientiane Capital and in two districts of Bolikhamxay province, respectively, based on numbers of students and location (Supplementary Figure 1). Students between the ages of 11 and 18 years were randomly selected with respect to the school grade and gender in order to have the same age and gender ratios in both provinces. In total, 388 students were enrolled in Vientiane Capital (urban), 197 students in Paksan, the Capital of Bolikhamxay (urban) and 194 students in Pakkading district (semi-urban). All parents/guardians signed the informed consent form and participants could withdraw their participation at any time. Age, gender, vaccination history, history of liver disease in the family and other potential risk factors were collected digitally on a tablet using Kobotoolbox (KoBoToolbox, 2020b). Since some of the participants were uncertain about their date of birth, the birth year was calculated by subtracting the age that they stated in the questionnaire from the year of sample collection. As a control, birth dates were matched with calculated birth years and the discrepancy was >1 year only for less than 5% of the participants. Data from the current study were compared to data collected in 2011 (Black et al., 2014). In the 2011 study, 560 school children aged 5–19 years participating in a 2011 national immunization campaign against measles and rubella were recruited. From this cohort, we selected 386 students from Bolikhamxay between 11 and 18 years old for comparison to the 2018 data. The selected students were from the same districts. 48.8% and 50% of the participants were male in 2011 and 2018 respectively. No participants were recruited from Vientiane Capital in 2011 in this age group.

Laboratory analyses

After informed consent, 5 ml blood was collected from each participant by a health care worker. Serum was separated by centrifugation on the day of the sample collection and stored at −20 °C for a maximum of two months, before being transferred to long-term storage at −80 °C. All samples were tested on the day of the sample collection with a rapid test for the hepatitis B surface antigen (HBsAg; ONE STEP HBsAg TEST, SD BIOLINE), which is a marker for chronic infection. Results and information regarding HBV infection were handed out in sealed envelopes to the students for their parents. In case students were found to be positive for HBsAg, they were recommended to seek medical advice and to confirm the rapid test result. Commercial ELISA kits were used to determine anti-HBs (quantitative; Diasorin) and anti-HBc (qualitative; Diasorin) antibodies. The presence of anti-HBs antibodies in absence of anti-HBc antibodies indicates protection through vaccination, while the presence of anti-HBc antibodies indicates infection with HBV. Anti-HBc positive and anti-HBs negative sera were tested for HBsAg (qualitative; Diasorin). An anti-HBs titer ≥10 IU/L was considered as protective (Jack et al., 2002).

The serum samples collected during the study in 2011 were tested with the same commercial ELISA kits (Diasorin, Italy).

Statistical analyses

Data analyses were conducted using R software (R Core Team, 2017) with the following packages: "stats" (R Core Team, 2017), "MASS" (Venables et al., 2020c), "epitools" (Aragon, 2017), "fmsb" (Nakazawa, 2018), "lmtest" (Zeileis and Hothorn, 2020d), "rcompanion" (Mangiafico, 2016), "pROC" (Robin et al., 2011), "tidyverse" (Wickham, 2017) and "car" (Fox and Weisberg, 2020e). Data from 2018 was compared between Vientiane Capital and Bolikhamxay province for all age groups specified. Data from Bolikhamxay in 2018 was compared to data collected in 2011 from the same age group.

In order to identify variables potentially associated with the antibody status, bivariate analyses were performed. Chi-square test and Fisher's exact test were applied to assess which factors are associated with seroprotection. Odds Ratio (OR), 95% confidence interval (CI) and p-values were calculated. Only variables with p-values <0.2 were included in the models. A correlation value >0.5 or a variance inflation factor >5 was considered as correlation and the decision to exclude the variable was made on a case-by-case basis: the variable which was deemed to be less important and/or with the lower impact was removed. Binary regressions were performed using a stepwise method for removing variables that were not associated with the response variable one by one, taking both the p-value of the variable and the Akaike Information Criterion (AIC) of the model into consideration. The significance of the final model in comparison with the null model was tested using a likelihood ratio test (R function "anova" with argument test set to "Chisq"). In addition, the individual effect of the variables in the model was tested by Wald tests (R function "Anova" with argument test.statistics set to "Wald").

Results

Participants in 2018

The majority of the students lived with their parents (85.9%). 46.9% of the students in Vientiane Capital and 50.6% in Bolikhamxay were male (Supplementary Table 1). In Vientiane Capital, nearly half of the students (47.7%) were born in a Central hospital in the Capital and only 7.5% were born at home. Nearly all students were of Tai-Kadai ethnicity (98.5%). In Bolikhamxay province, only 2.8% of the students were born in a Central hospital, 16.1% were born in a Provincial hospital and more than one third (38.1%) of the participants was born at home. In the province, 7.2% of the students were of Hmong-Mien, Mon-Khmer or another non-Tai-Kadai ethnicity. A large proportion of the students did not know their place of birth (26.6% in Bolikhamxay, 37.9% in Vientiane Capital). None of the participants stated to have used intravenous drugs and only two – in Vientiane Capital – stated to have a tattoo.

2.6% of all students had received a blood transfusion and 6.3% stated that they were aware of chronic liver disease in their family. The questions regarding sexual relationships were only asked to students >13 years. In both provinces, 13.5%-14% stated to have had a sexual relationship starting at the age of 14 years. About half of those with a previous relationships stated to have had more than one partner in the past 6 months. Documentation of previous vaccinations, such as the yellow immunization cards, were not available for all but three students.

Serological profiles

Overall serology profiles

In 2018, the overall anti-HBc seroprevalence was 6.4% and 12.6% in Vientiane Capital and Bolikhamxay province, respectively ($p < 0.01$, Table 1). In Bolikhamxay, this is a significant decrease compared to the same age group in 2011 (19.2%, $p < 0.05$) — all born before vaccine introduction (Black et al., 2014). In addition, the HBsAg prevalence in 2018 was significantly lower in Vientiane Capital than in Bolikhamxay (1.6% vs. 4.4%, $p < 0.05$, Table 1). However, there was no statistical difference between Bolikhamxay in 2018 and 2011, when 3.4% of the students tested positive for HBsAg. The serological profile typical for vaccination (anti-HBs +/anti-HBc−) was only somewhat higher in Vientiane Capital than in Bolikhamxay in 2018 (19.3% vs 15.4%), and the difference was not significant (Table 1). The proportion of students negative for both anti-HBc and anti-HBs was similar in Vientiane and Bolikhamxay and increased significantly in Bolikhamxay from 61.4% in 2011 to 72.1% in 2018 ($p < 0.01$).

Serological profiles by birth cohort

HBV vaccination was gradually introduced into the national immunization programme from 2001 onwards (Black et al., 2014; Phoummalaysith et al., 2018; Xeuatvongsa et al., 2014; Kolwaite et al., 2016). Figure 1A and B show the serological profiles by year of birth in Vientiane capital and Bolikhamxay province in 2018.

In Vientiane, there is a visible increase in the serological profile for vaccination already in 2003, while in Bolikhamxay it took a year longer (Figure 1). If we compare serological markers for vaccination (anti-HBs+/anti-HBc−) before and after these dates, we find an increase of 12.2%–25% ($p < 0.01$) in Vientiane and from 3.4%–28.5% ($p < 0.00001$) in Bolikhamxay. The decline of anti-HBc(+) students was also significant at both recruitment sites between these years (9.3% vs. 4.2%, $p < 0.05$ in Vientiane Capital and 18.1% vs. 6.5%, $p < 0.001$ in Bolikhamxay). In Bolikhamxay province, but not in Vientiane, also the decrease of HBsAg(+) students was significant (6.4% vs. 2.1%, $p < 0.05$). There was no statistical difference between the proportion of students with the serological vaccination profile born after the visible impact of the vaccine in Vientiane Capital (2003–2007) and Bolikhamxay Province (2004–2007) (28.5% in Bolikhamxay vs. 25% in Vientiane Capital, $p = 0.43$).

Even when only those students born in or before 2001, the year of the introduction of the vaccine, were compared to those students born after this date, the prevalence of students with serological evidence of vaccination increased from 13.2% to 21.9% ($p < 0.05$) in Vientiane and from 3.0% to 19.7% ($p < 0.001$) in Bolikhamxay. At the same time, serological evidence of previous infection declined from 11.4% to 4.4% in Vientiane ($p < 0.05$) and 22.0% to 9.3% ($p < 0.01$) in Bolikhamxay. The prevalence of HBsAg did not decrease significantly in Vientiane or Bolikhamxay.

When students of the same age (11–14 years old) from Bolikhamxay in 2011 (Figure 1C) were compared to 2018, the proportion of students with a serological vaccination profile was considerably higher in 2018, after the introduction of the vaccination (28.5% vs. 17.6%, $p < 0.05$). The proportion of students that were HBc(+) was also dramatically lower in 2018 compared to 2011 (6.5 vs. 20.1%, $p < 0.001$). However, the prevalence of HBsAg (+) students was not significantly different in 2018 in this age group (6.5% in 2018 vs. 6.2% in 2011, $p = 0.56$).

The prevalence of the vaccination profile was around 25–30% in Bolikhamxay province in the 2011 data in participants born in 2002–2004 as compared to 3.5–22.6% in participants born in the same years in the 2018 study (Figure 1). Since participants born in 2002–2004 were 14–16 years old by 2018, this discrepancy is likely due to antibody waning over time.

Bivariate analyses of 2018 data

Bivariate analyses were performed on the 2018 data to investigate factors associated with the typical vaccination serology as well as HBV infection. In 2018, age >13 years, not living with parents, not born in a central hospital and being in an upper school class were negatively associated with the vaccination serology while recall of HBV vaccination was positively associated ($p < 0.05$). All variables associated with the vaccination profile with a $p < 0.2$ were included in the regression analyses, including in addition province, number of household members, history of chronic liver disease in the family and more than one sexual partner in the last 6 months (Table 2).

Exposure to HBV (anti-HBc+) was significantly associated ($p < 0.05$) with higher age, living in Bolikhamxay province, living in Pakkading district, not living with parents, not born in Vientiane, higher school grade, a history of chronic liver disease in the family, and lower level of the nearest health care facility (HCF) (Table 2). In addition, gender, number of household members, history of sexual contact and >1 sexual partner in the past 6 months were associated with exposure to HBV with a $p < 0.2$ and were included in logistic regression analyses.

Table 1
HBV profiles of students aged 11–18 years according to study year and study site.

HBV serology	2018				P-value[a]	2011		P-value[b]
	Vientiane Capital		Bolikhamxay			Bolikhamxay		
	n	%	n	%		n	%	
Anti-HBs (−)/anti-HBc (−)	288	74.23	281	72.05	0.49	237	61.40	<0.01
Anti-HBs (+)/anti-HBc (−)	75	19.33	60	15.38	0.15	75	19.43	0.14
Anti-HBs (+)/anti-HBc(+)	15	3.87	25	6.41	0.11	29	7.51	0.54
Anti-HBs(−)/anti-HBc(+)	10	2.58	24	6.15	<0.05	45	11.66	<0.01
Total	388		390			386		
All HBsAg (+)	6	1.55	17	4.36	<0.05	13	3.37	0.47

[a] Comparison between Vientiane Capital and Bolikhamxay province in 2018.
[b] Comparison between 2011 and 2018 data in Bolikhamxay province.

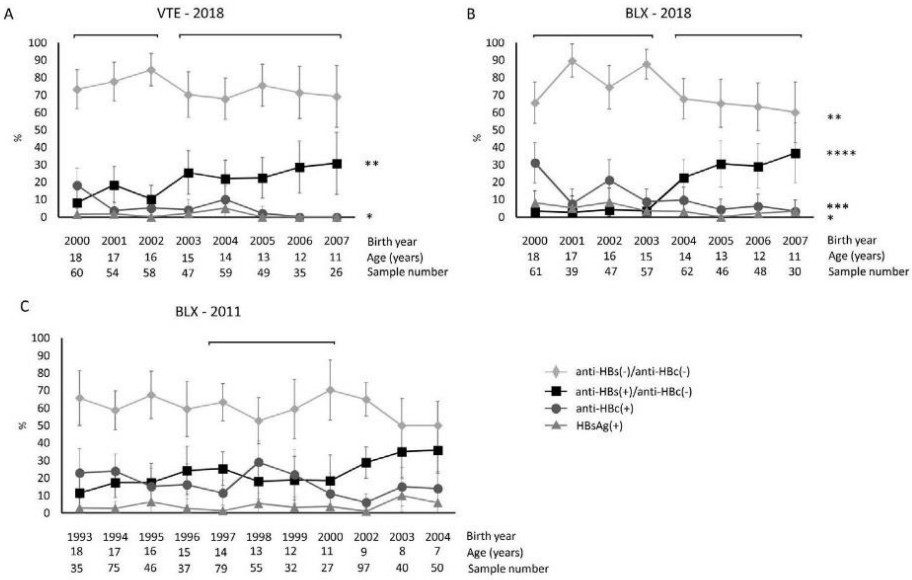

Figure 1. Hepatitis B serological profiles in (A) Vientiane Capital in 2018, (B) Bolikhamxay province in 2018 and (C) Bolikhamxay province in 2011. Data points are presented with 95% CIs. * = p < 0.05; ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001. In panel A, * relates to the line corresponding to the prevalence of anti-HBc(+), in panel B, * relates to the line corresponding to the prevalence of HBsAg. In panel C, data from 2001 was omitted due to low sample numbers. The brackets above the serological profiles indicate the birth year cohorts that were compared with each other. Abbreviations: VTE, Vientiane; BLX, Bolikhamxay.

Logistic regression analyses

Some variables with low numbers (living situation, recall of HBV vaccination and the number of sexual partners) were excluded from the multivariable modelling for identifying factors independently associated with vaccination serology. Since a large proportion of the students did not know their place of birth, this variable was also excluded. School grade was excluded since it correlated with age.

Students who were born earlier and recruited in Bolikhamxay were considerably less likely to have a serological profile of vaccination against HBV (interaction term in model; p = 0.003, OR = 0.74, CI = 0.60–0.90) (Table 3). The overall model was significant (p < 0.0001) but the effects of all variables in the final model could only explain 12% of the variability in the dataset (Nagelkerke Pseudo-R^2).

The final multivariable model of risk factors for HBV infection is shown in Table 4. The same variables were excluded for the reasons detailed above. The variables describing province and district also contained the same information and were individually tested in the models. Students living in Pakkading rather than Vientiane Capital (p < 0.0001) (Table 4) and older students (i.e. born earlier) as well as students with known history of chronic liver disease in their families were more likely to be positive for anti-HBc (p < 0.0001 and p = 0.003 respectively). The overall model was significant (p < 0.0001), but the effects of all variables in the final model could explain only 15% of the variability in the dataset (Nagelkerke Pseudo-R^2).

Discussion

The proportion of participants with a serological vaccination profile increased only moderately but significantly after the introduction of HBV vaccination into the national vaccination programme in both Vientiane Capital and Bolikhamxay province. Logistic regression analyses confirmed that the odds of being protected against HBV through vaccination decreased with age in combination with living in Bolikhamxay province. These results are in line with the somewhat delayed extension of the HBV vaccinations from the Capital to the provinces.

In Bolikhamxay, the vaccination seems to have caught on only in children born and vaccinated in 2004, one year later than in Vientiane. Nevertheless, in both study sites, only about 30% of the participants born 5–6 years (e.g. in 2006–2007) after the introduction of HBV vaccination showed the anti-HBs(+)/anti-HBc(−) vaccination profile, up from pre-introduction levels of 8.3% in Vientiane and 3.3% in Bolikhamxay.

Since this study was done in 2018, more than 10 years after introduction of the vaccine, these rates reflect vaccine coverage, primary and secondary vaccine failure as well as waning of anti-HBc antibodies after a resolved HBV infection. It is likely that vaccine coverage was initially low and only increased with time to the 85% levels reported in 2017 for the entire country (WHO, UNICEF, 2018). In addition, in another study we found that the response to the hepatitis B component of the pentavalent vaccine in children vaccinated with 3 documented doses in 2009–2012 was below 40% within 9–50 months after vaccination, with a considerable waning effect (primary and secondary vaccine failure) (Evdokimov et al., 2017).

In the present study, the relatively modest increase in anti-HBs (+) translated nevertheless into a 2–3 times lower prevalence of markers of resolved (anti-HBc positive) and chronic infection (at least in Bolikhamxay), possibly showing some evidence of less horizontal transmission due to vaccination efforts. Our findings are comparable to those of other studies observing decreasing anti-HBc positivity rates along with increasing estimated serological vaccination rates after the introduction of HBV vaccination (Su et al., 2007; Fujimoto et al., 2018). Furthermore, in logistic regression, older students and students living in Pakkading district, a semi-urban area, were significantly more likely to show evidence of past infection with HBV.

However, even among the youngest students between 11–13 years old, 60%–75% did not have any serological evidence of successful immunisation. Some of these individuals who may have

Table 2
Factors associated with being seroprotected against HBV (anti-HBs+/anti-HBc−) and exposure to HBV (anti-HBc+).

Variables[a]	Categories	N/group	Anti-HBs+/anti-HBc−				Anti-HBc+			
			% protected	OR	95% CI	p	% positive	OR	95% CI	p
Province	Vientiane Capital	388	19.33	1			6.44	1		
	Bolikhamxay	390	15.38	0.76	0.52–1.10	0.1562	12.56	2.09	1.26–3.45	0.0047
District	Vientiane Capital	388	19.33			NS	6.44	1		
	Paksan	197	14.72				8.12	1.28	0.67–2.46	0.4941
	Pakkading	193	16.06				17.1	2.99	1.72–5.20	0.0001
Living situation of students	With parent(s)	668	18.86	1			7.34	1		
	Other	110	8.18	0.38	0.19–0.78	0.0043	22.73	3.72	2.18–6.33	<0.0001
Gender	Male	379	17.15			NS	11.08	1		
	Female	399	17.54				8.02	0.7	0.43–1.13	0.1784
Age of participants	≤13 years	234	29.06	1			2.99	1		
	14–16 years	330	14.85	0.43	0.28–0.64	0.0001	9.7	3.48	1.51–8.03	0.0021
	>16	214	8.41	0.22	0.13–0.39	<0.0001	16.36	6.34	2.75–14.61	<0.0001
Place of birth	Central Hospital in Vientiane Capital	195	23.08	1			5.13	1		
	All other places and "I do not know"	583	15.44	0.61	0.41–0.91	0.0165	10.98	2.28	1.15–4.54	0.0160
Nearest HCF[b]	CH and PH	394	18.53			NS	6.85	1		
	DH and HC	384	16.15				12.24	1.9	1.15–3.11	0.0141
School grade	Lower secondary	444	24.77	1			6.31	1		
	Upper secondary	334	7.49	0.25	0.15–0.39	<0.0001	13.77	2.37	1.45–3.89	0.0005
Recall of HBV vaccination	No	762	16.93	1			9.58			NS
	Yes	16	37.50	2.94	1.05–8.24	0.0433	6.25			
Number of household members	≤4	257	21.01	1			7.39	1		
	>4	521	15.55	0.69	0.47–1.01	0.0697	10.56	1	0.86–2.55	0.1936
History of chronic liver disease in family	No & I do not know	729	16.87	1			8.64	1.48		
	Yes	49	24.49	1.6	0.81–3.15	0.1746	22.45	3.06	1.49–6.28	0.0041
History of sexual relationships (only students > age 13 years were asked)	No	469	13.01			NS	11.3	1		
	Yes	75	8.00				18.67	1.8	0.94–3.44	0.0870
More than one partner in the past 6 months (sexual relationship)	No	38	2.63	1			10.26	1		
	Yes	37	13.51	5.78	0.64–52.10	0.1075	30.77	3.15	0.89–11.15	0.0816

[a] Variables that were not significantly associated to either serological profile are not shown. These variables include: Vaccinations at school, receiving blood transfusion, number of received blood transfusion, age at first sexual contact, use of condom during sexual contact, ethnicity and travel time to nearest HCF.
[b] CH = Central Hospital, PH = provincial hospital, DH = district hospital, HC = health center.

Table 3
Binomial generalized linear model showing the factors affecting seroprotection against HBV.

Factors inlcuded in the final model	Summary of model					
	OR	95% CI	Estimate	Std. error	z Value	Pr(>|z|)
Intercept	0.23	0.18–0.30	−1.46	0.13	−10.99	0.0000
Province (Bolikhamxay)	0.51	0.32–0.80	−0.67	0.23	−2.85	0.0044
Age	0.83	0.73–0.93	−0.19	0.06	−3.07	0.0021
Age: Province (Bolikhamxay)	0.74	0.60–0.90	−0.30	0.10	−2.94	0.0033

lost their anti-HBs antibodies despite a previous vaccination may be protected through immune memory (Simons et al., 2016; Wenzel and Jilg, 2010; Brunskole Hummel et al., 2016; Carollo et al., 2013; Zanetti et al., 2010). The higher anti-HBc seroprevalence in the older age group may also be partially due to a higher accumulated risk of exposure as the adolescents get older.

Our logistic regression models showed that there were few predictors of past infection or seroprotection against HBV, indicating that there were few systematic risk factors besides age and location. The relatively low Pseudo-R^2 values indicate a high variability in the dataset.

Although we can detect these beneficial effects in this early birth cohort, today the seroprevalence of protective anti-HBs antibodies is too low in these adolescents and warrants at least a refresher booster before they enter into a phase of their lives with new risk factors.

Unfortunately, we were not able to assess the association of certain risk factors with the serological profiles due to low numbers. Increased exposure to risk factors for older students and changes in social behaviour may lead to potential health hazards in the future in Lao PDR.

When the serological profiles in 2018 were compared to data from 2011 from Bolikhamxay province, the proportion of students exposed to HBV decreased significantly in the age groups 11–14 years, although the prevalence of HBsAg, or chronic infection, did not. This discrepancy may be due to the slow uptake of the birth dose, which even in 2017 was only at 55% nationwide. While the coverage with the pentavalent vaccine (at 6, 10 and 14 weeks) has steadily increased to 85% in 2017 and was effective in reducing infections later in life, it seems to have done little to prevent perinatal vertical transmission and the associated high risk of chronic disease. Indeed, a history of chronic liver disease in the

Table 4
Binomial generalized linear model showing the factors associated with being exposed to HBV.

Factors inlcuded in the final model	Summary of model					
	OR	95% CI	Estimate	Std. Error	z Value	Pr(>|z|)
Intercept	0.00	0.00–0.00	−8.32	1.12	−7.41	0.0000
District (Paksan in Bolikhamxay)	1.48	0.75–2.87	0.39	0.34	1.15	0.2505
District (Pakkading in Bolikhamxay)	3.44	1.95–6.18	1.24	0.29	4.21	0.0000
Age	1.42	1.25–1.63	0.35	0.07	5.21	0.0000
Incidence of chronic liver disease in family	3.17	1.42–6.63	1.15	0.39	2.96	0.0031

family was associated with serological evidence of previous infection in our study. These findings underline the importance of further increasing the coverage of the birth dose.

Since we only included students from two provinces, our ability to extrapolate the findings to the whole country is limited. In addition, we only included students from urban and semi-urban areas that belonged mainly to the Tai-Kadai ethnicity. Since vaccination documents for the students in both studies were unavailable to us, we could not interpret the findings with respect to vaccination status or the number of hepatitis B vaccinations. Another limitation of this study is that the risk factor history relied largely on self-reporting.

In conclusion, this study documented a sizable and lasting reduction in past HBV infections in adolescents born early after the introduction of infant HBV vaccination. However, the combination of low coverage and supposedly low immunogenicity resulted in low overall protective levels, which may warrant the introduction of a booster for adolescents at least of these intermediary birth cohorts. Furthermore, additional research may be needed to determine if a booster vaccination is enough to protect these adolescents. A combination of an HBsAg containing vaccine with the planned introduction of the school-based HPV vaccine may be considered. The effect of routine infant vaccination on chronic infection is not measurable, probably because the uptake of the birth dose was very low in these both cohorts. There is therefore an urgent need to increase the birth dose coverage significantly higher than the 55% reported in 2017. In addition, awareness campaigns regarding the risk factors of HBV infection should be conducted.

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Conflict of interest

Dr. Nouanthong has been a member of the independent National Immunization Technical Advisory Group in Lao PDR since August 2017. The other authors declared no conflict of interest.

CRediT authorship contribution statement

Lisa Hefele: Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Souphaphone Vannachone:** Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Vilaysone Khounvisith:** Conceptualization, Data curation, Writing - original draft, Writing - review & editing. **Phonethipsavanh Nouanthong:** Conceptualization, Writing - original draft, Writing - review & editing. **Somphou Sayasone:** Conceptualization, Writing - original draft, Writing - review & editing. **Sengchang Kounnavong:** Conceptualization, Writing - original draft, Writing - review & editing. **Phetsavanh Chanthavilay:** Conceptualization, Writing - original draft, Writing - review & editing. **Claude P. Muller:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing. **Antony P. Black:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2020.01.055.

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CHAPTER 9. Discussion and conclusion

9.1. Discussion

Each of the different studies has been discussed in the previous chapters. Here, I briefly restate and discuss the main findings of this research and their implications for vaccine policy in the context of the Lao PDR. In addition, strengths and limitations of the studies will be reviewed.

One of the research objective of this thesis was it to characterize the epidemiology of VPDs before and after the introduction of routine vaccination. When I started these studies, data regarding the seroprevalence of *H. influenzae* type b and *S.* Typhi in the Lao PDR were lacking. The first study in this thesis (**CHAPTER 2**) aimed to contribute to the understanding of *S.* Typhi seroprevalence in the general population in Lao PDR.

In 2018, the MoH of the Lao PDR initiated a typhoid fever disease burden assessment to evaluate the benefit of introducing a typhoid conjugate vaccine. For the introduction of country-wide vaccination programme, a number of issues need to be considered including the disease burden, performance of available vaccines, supply logistics, public perception or cost-effectiveness. Financial aspects of vaccine introduction are also critical for the decision as Lao PDR´s country income exceeded the eligibility threshold for GAVI support in 2017. By 2021/22, the country is expected to fully self-finance its vaccination programmes[135].

From July to August 2019, the US-CDC conducted an initial assessment of typhoid disease burden. A national "Stakeholders Consultation Meeting on Typhoid Fever Prevention and Control in Lao PDR" was organized by the MoH in October 2019. At this time, our study was on-going and only preliminary findings were presented. After the initial assessment of data from various research groups in the Lao PDR, it became clear that the current data situation on typhoid fever in the country was inconclusive at that time. Therefore, the CDC recommended to conduct a systematic evaluation of the typhoid fever surveillance and laboratory capacity for blood culture, to review cases of non-traumatic intestinal perforations and to consider water, sanitation and hygiene strategies [116]. Increased understanding of the local typhoid fever epidemiology will help designing a national typhoid fever vaccination programme.

Recent studies have explored serological markers for typhoid fever exposure. Both anti-HlyE IgG and anti-CdtB IgG have been previously suggested as useful biomarkers to distinguish typhoid from non-typhoid fever cases [5,13,104,118]. Other studies focus on antibody responses against the immunogenic polysaccharide capsular Vi antigen, the prime target for vaccine development [30,42,126].

Our data provided the first serological assessment of the age-stratified seroprevalence of anti-*S.* Typhi antibodies to serve as a baseline for further outbreak and surveillance studies. The prevalence of

antibodies developed against the two protein antigens was higher in children than in adults, with a peak in children <5 years of age. In contrast, the serological profile of anti-Vi IgG correlated clearly with increasing age. Since anti-HlyE and anti-CdtB antibodies may be more suitable makers for *S*. Typhi exposure, as they are developed against protein antigens, our results suggest a high exposure in children. Introducing TCV vaccination for children may proof beneficial for the country, although more data is needed to adequately describe typhoid fever epidemiology. Follow-up studies could investigate how water, sanitation and hygiene interventions impact typhoid fever incidence. Including children from several provinces and age groups could provide more information on typhoid fever disease burden in different areas of the Lao PDR. Thailand and Vietnam are examples of effective typhoid fever control strategies including the vaccination of children. In response to typhoid fever outbreaks in the 1970s, Thailand created a typhoid immunization programme targeting over 5 million school children[24,114]. A recent study reports a general decline in typhoid fever incidence in Thailand over the past 20 years [114]. However, regional variability still exists and outbreaks continue to occur periodically. In Vietnam, typhoid fever was probably endemic before the turn of the century, but incidence data is only available as of 1999. Vaccination campaigns were initiated as early as in the 1990s and targeted mostly children. In 2005, up to 35 provinces of Vietnam were part of the national typhoid Vi vaccine immunization campaign. Today, the incidence of enteric fever in Vietnam is estimated to be very low [76].

Our study investigating the prevalence of *H. influenzae* type b (**CHAPTER 3**) revealed that all unvaccinated individuals showed serological evidence of natural exposure before the introduction of a vaccine, thus confirming the need for the vaccination. We also observed antibody waning after vaccination emphasizing the need for the strengthening of the vaccine management.

Both the studies in **CHAPTER 2** and **CHAPTER 3** represent first baseline studies of Hib and typhoid fever epidemiology. In addition to the serological data, clinical data from all provinces are required for the understanding of *H. influenzae* type b and typhoid fever disease burden.

Both studies have limitations; samples were initially collected in the framework of different studies with different designs, somewhat undermining the representativeness. While there is a correlate of protection for Hib, the interpretation of the antibodies against various typhoid antigens remains complicated.

At the moment, the policy briefs for local partners, policy-makers and public health organisations in the Lao PDR are being prepared. Our recommendations include:

1. Implementing a robust Hib and typhoid fever surveillance system based on laboratory confirmation
2. Strengthening vaccine management to achieve sufficient levels of protection against *H. influenzae* type b

The performance of vaccination programmes is often monitored through tracking vaccination coverage data, but under- or over-reporting directly impacts these data and provides a distorted picture. In the Lao PDR, data regarding vaccination coverage rates vary depending on the source of information. The official country coverage rates for the pentavalent vaccine in the years 2017, 2018 and 2019 were reported as 85%, 84% and 92%, respectively. However, the WHO and UNICEF estimates for the same year were considerably lower with 69%, 68% and 68%, respectively[155]. Both country estimates are below vaccination coverage rates for DTP3 (the third dose of the DTP containing vaccine) in the WHO Western Pacific Region, which were estimated above 90% for the same years[32]. The assessment of progress of the NIP is complicated by inconsistent data. Furthermore, in countries like Laos, crude immunization coverage (proportion of individuals who received the vaccine) may not correlate well with antibody seroprevalence because of low seroconversion as a result of poor vaccine management. For example, low vaccine immunogenicity after full vaccination with the pentavalent vaccine was observed in a previous study by the LaoLuxLab in 2013/14 without clear explanation [28]. Vaccination coverage data could overestimate population immunity, particularly in countries where reporting data quality is unsatisfactory. Thus, seroepidemiology can help designing vaccination campaigns, identify immunity gaps in the population and determining the duration of immunity.

In **CHAPTER 4**, **CHAPTER 5** and **CHAPTER 6**, serology data was used to monitor and evaluate immunization programme performance. Two vaccines were investigated: the pentavalent DTP-HepB-Hib vaccine and the MR vaccine.

CHAPTER 4 and **CHAPTER 5** describe a follow-up study conducted in 2017 which investigated the immunogenicity of the DTP-HepB-Hib [40] and the MR vaccine, respectively. Serum samples from children fully vaccinated with DTPw-HepB-Hib were collected from one province in central Lao PDR and Vientiane capital and tested for antibodies against diphtheria, tetanus, pertussis, HBV, Hib, measles and rubella, as well as for serological markers of infection with HBV. A special focus was given to the site/level of health care system on which the vaccine was administered. Compared to the earlier findings in Bolikhamxay, seroprotection rates improved considerably in 2017 compared to 2013/14 but remained mostly suboptimal: Seroprotection rates in vaccinated children against tetanus increased from 53.6% to 75.2%, for diphtheria from 55.2% to 77.3%, for Hib from 66.4% to 71.7% and for hepatitis B from 37.7% to 72%. Similarly, the proportion of vaccinated children being positive or borderline for anti-measles increased from 65.8% to 76.5% and from 82.6% to 95.6% for anti-rubella. The prevalence of anti-HBc, a marker for past exposure to hepatitis B decreased from 13.2% to 3.1%.

In logistic regression analyses, the travel time from HC to DH was identified as a risk factor for low seroprotection rates against hepatitis B, diphtheria and tetanus[40]. In the logistic regression model assessing factors associated with seropositivity for measles and rubella antibodies, the distance to the nearest HCF and vaccination at a HC were retained in the best fitting model, albeit not being significant.

These findings underline the need to strengthen vaccine management in particular at the lower levels of the health care system.

In Bolikhamxay, only 76% of the children vaccinated with MR had an anti-measles and anti-rubella IgG titer corresponding to being positive or borderline. Up to 20% were only positive for anti-rubella IgG. The variance between the two vaccine response rates could indicate different stabilities between the two vaccine components, as previously described by [124]. Furthermore, a high percentage of children who did not yet receive the MR vaccine were nevertheless positive for antibodies against measles and rubella, indicating natural exposure through virus circulation, underreporting of cases and/or problems with vaccination documentation.

The following recommendations were made to public health authorities in the Lao PDR (policy briefs in Appendices **D.a** and **D.b**):

1. Conducting a review and strengthening of the vaccine management with a focus on remote areas
2. Including an additional booster dose with DTPw-HepB-Hib, to boost the seroprotection of those that had already received all three doses, but also to act as a catch-up for those children that missed one or two of the earlier doses
3. Strengthening the measles and rubella surveillance system by systematic laboratory testing for improved reporting

Vaccination coverage and vaccination timeliness are separate issues but they are interconnected. Delayed vaccination increases the period in which the child is susceptible to infection and may facilitate disease outbreaks. High vaccination coverage rates may hide vaccination delays which could undermine the effectiveness of vaccination programmes.

The data obtained during the seroprevalence studies were used to assess timeliness of vaccination with DTPw-HepB-Hib (**CHAPTER 6**). In general, vaccination with the pentavalent vaccine was delayed, especially the third dose and especially in health care settings below the provincial level. Considerable discrepancies between yellow immunization cards and hospital records were observed. Vaccination delay was independently associated with lower education of the mother, not receiving the hepatitis B birth dose and vaccination by outreach service. Vaccination timeliness also may have an impact on immune response. Unfortunately, since most of the participants were not vaccinated earlier than recommended or within shorter time intervals between doses, we could not investigate a possible impact of premature vaccination on seroprotection.

The recommendation to public health authorities in the Lao PDR included (Appendix **D.c**):

1. Providing further training about the importance of timely vaccination to the outreach teams

2. Organizing outreach vaccination according to a sustainable and predictable schedule
3. Including timely completion of the vaccination series as a quality indicator for the NIP
4. Monitoring completion and timely completion as a surrogate measure for general access to health care
5. Investigating the reasons for the discrepancies and inconsistencies in recorded dates
6. Providing training for health care workers about the importance of robust documentation of vaccination and the timeliness of vaccination

Limitations of the studies in **CHAPTER 4**, **CHAPTER 6** and **CHAPTER 5**, include their regional focus on Central Lao PDR which impacts their generalization to other regions and the whole country. Serology only sometimes allows the distinction between vaccine-induced protection and natural infection (for example in the case of hepatitis B vaccination). The place of vaccination could only be obtained from parental recall. Seroprotection levels of antibody titers have been well established for hepatitis B, diphtheria, tetanus and Hib, but not, for example, for pertussis. If no clear correlation of protections exists with vaccine responses, antibody titer data needs to be interpreted with more caution. Furthermore, while the serological response to vaccination is indispensable for assessing immune responses, they are only one component of the immune defence system, but even quantitative measurement of vaccine-induced immune cell memory can normally not be related to protection. In order to further investigate the vaccine-induced immune cell memory in addition to the antibody responses, other laboratory techniques (for example enzyme-linked immuno-spot assay) need to be employed for the quantification of specific T- or B-cell responses. Lastly, the study findings cannot be used to derive vaccination coverage rates or the proportion of children who missed vaccination doses since only children with a full course of DTPw-HepB-Hib were included.

Further investigations should be conducted to investigate low vaccine responses in other regions or in other ethnic groups of the Lao PDR. A thorough assessment of structural factors in the health care system that contribute to problems with the vaccination management may reveal risk factors beyond the individual´s socio-economic, behavioural or health-related characteristics. In addition to investigating the seroprotection levels, other existing data resources, such as for example the Multiple Indicator Cluster Surveys, could be mined to investigate trends in coverage rates, socio-economic factors related to the coverage rates or full vaccination status, or regional or seasonal variations. More information is also needed on missed opportunities for vaccination in the Lao PDR, maternal knowledge and perceptions about immunization. Longitudinal serology studies could also provide insights into the dynamics of vaccine-induced antibodies and provide information regarding antibody waning or continued natural boosting.

In **CHAPTER 7** and **CHAPTER 8**, the current knowledge regarding HBV epidemiology in the Lao PDR and the impact of hepatitis B vaccination in a randomly selected population of adolescents was assessed.

A literature review regarding hepatitis B epidemiology in the Lao PDR (**CHAPTER 7**) provided an up-to-date summary of serological data in the Lao PDR (please note that in the literature review, the study regarding the impact of hepatitis B vaccination was also included since the research study manuscript was published before the review manuscript). The reviewed studies varied widely regarding study design and methods. Our ability to draw conclusions from the past study findings is limited due to this heterogeneity in research approaches but also due to the ethnic and geographic diversity in the Lao PDR. Chronic infection rates are still high and continue to be a major public health challenge in the future. Despite often weak vaccine responses, vaccination efforts may likely reduce the prevalence of chronic HBV infection in the country, as two studies reported first successes in terms of improvement of seroprotection and impact of vaccination [40,41].

The results of our serological study regarding hepatitis B (**CHAPTER 8**) showed a considerable reduction in past HBV infection among adolescents in Vientiane Capital and Bolikhamxay province. Overall, the HBsAg prevalence was 1.6% in Vientiane Capital and 4.4% in Bolikhamxay province. HBsAg prevalence decreased in Bolikhamxay significantly from 6.4% to 2.1% after the introduction of the vaccine. However, vaccine-induced seroprotection levels among the participants were relatively low, ranging from 15.4% in Bolikhamxay to 19.3% in Vientiane Capital.

A current report about chronic hepatitis B infection in the Western Pacific Region (WPR) documented the progress made towards hepatitis B elimination [134]. In 2017, 53% of the 36 countries in the WHO WPR were reported to have achieved <1% HBsAg prevalence target. 42% and 50% of the countries met the vaccination coverage goals of ≥95% for the hepatitis B birth dose and the third dose of hepatitis B vaccination. Both Vietnam and Cambodia did not yet meet these targets [134]. Thailand, a country belonging to the WHO South-East Asia Region, did meet the <1% HBsAg prevalence target as of September 2019 [145]. Based on available HBV data for Lao children under 5 years of age, it is unlikely that the target of reducing the HBsAg prevalence under 1% will be met in the near future. Further investigations of Hepatitis B prevalence in the Lao PDR should aim on identifying the challenges for the hepatitis B birth dose coverage and serosurveys aiming on determining the current hepatitis B burden in all age groups in the population should include both sexes.

Main limitations of our study include the geographic focus on the Central regions in Lao PDR[41]. Ethnic groups other than the Tai-Kadai ethnicity were underrepresented in the study. Vaccination documents were unavailable and information of risk factors in the family relied on recall.

The study findings were presented to public health authorities in the Lao PDR and included the following recommendations (Appendix **D.d**):

1. Administration of an additional hepatitis B vaccine dose for adolescents
2. Implementation of awareness campaigns regarding the risk factors of HBV in high schools
3. Strengthening of routine infant HBV vaccination, especially of the HBV birth dose

Overall limitations and strengths of this thesis

This doctoral thesis described my sero-epidemiological investigations of VPDs and their implications for vaccination policy in the Lao PDR. There are several limitations: Some aspects of sero-epidemiological surveillance are not addressed in the research presented in this thesis, including the investigation of changes in disease strains or the detection of VPDs outbreaks and new pathogens. As discussed before in section **1.7**, it is only possible in certain instances, for example hepatitis B, to differentiate between naturally induced and vaccine-induced immunity. Antibody waning may also complicate the interpretation of serological results. Most studies focused on the Central regions of Lao PDR and the heterogeneity of the country makes it more difficult to generalize our findings. Although our logistic regression models were significant, the low Nagelkerke Pseudo-R^2 suggests that they could explain only a certain amount of the variability in the datasets and that some factors may simply not have been captured with our study designs. Study planning, study findings and their interpretation are based on, or should be seen in the context of existing data. It is important to keep in mind that the quality of official data itself in the Lao PDR may be limited.

Even with these limitations, our findings have important implications for public health practice. Serology is a powerful tool for the surveillance and monitoring of the prevalence of VPDs or vaccine-induced protection levels. The persistence of antibodies is useful for detecting infections even if they occurred in the past or went unnoticed. In the framework of this thesis, two field studies were conducted to collect serum samples and socio-economic and health-related data in order to investigate questions related to disease prevalence and vaccine-induced protection. The serum samples contributed to the growing serum sample biobank at the Institut Pasteur du Laos and can also be utilized in future research studies. Our studies addressed specific knowledge gaps regarding important public health issues related to VPDs in Lao PDR and can serve as basis for further studies. The studies identified risk factors and revealed weaknesses of the vaccination programme. In addition, the studies also demonstrated significant improvements made over the past few years. Tangible recommendations for vaccine policy makers resulted from our studies in the Lao PDR.

9.2. Conclusion

This thesis investigated several research questions related to vaccine-preventable diseases in the Lao PDR, a lower-middle income country characterized by a great geographic and socio-economic heterogeneity.

In **CHAPTER 2** and **CHAPTER 3**, serological testing was used to establish baseline data for the prevalence of *H. influenzae* type b and *S.* Typhi in the Lao PDR in absence of routine vaccination. This data can serve as reference for further follow-up studies and, in case of *S.* Typhi seroprevalence, they are complementary to available clinical data in the country. Before the introduction of Hib vaccination in the Lao PDR, the prevalence of Hib was high. Our data on age-stratified exposure to *S*. Typhi indicates a considerable prevalence of anti-*S*. Typhi antibodies in children.

In **CHAPTER 4**, **CHAPTER 5** and **CHAPTER 6**, serological data were collected in order to evaluate the performance of the pentavalent and the MR vaccine in the immunization program. The results highlighted weaknesses in vaccine management and the need for booster vaccinations. Serological data showed effective protection through vaccination as an indicator of vaccination success in addition to coverage data. Furthermore, the timeliness of receiving the pentavalent and the MR vaccine was assessed and risk factors of delayed vaccination with the pentavalent vaccine were identified.

In **CHAPTER 7** and **CHAPTER 8**, the current understanding of the HBV epidemiology in the Lao PDR was summarized as a literature review and the impact of hepatitis B vaccination on disease burden was assessed through serology.

In conclusion, the research articles presented in this thesis contributed to the understanding of VPD epidemiology in the Lao PDR and the performance of current vaccination efforts. Serological data were used to answer several different questions concerning the progress in VPD control in the Lao PDR. The findings were translated into vaccine policy recommendations.

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ຂອບໃຈຫຼາຍໆ

APPENDIX

A. LIST OF MANUSCRIPTS

a. Published manuscripts

Hefele L, Syphan S, Xayavong D, Homsana A, Kleine D, Chanthavilay P, Nouanthong P, Xaydalasouk K, Phathammavong O, Billamay S, Xeuatvongsa A, Reinharz D, Muller CP, Black AP. *Seroprotection at different levels of the health care system after routine vaccination with DTPw-HepB-Hib in Lao PDR.* Clin Infect Dis. 2019 Feb 19. doi: 10.1093/cid/ciz143.

Hefele L, Vannachone S, Kounvisith V, Nouanthong P, Sayasone S, Kounnavong S, Chanthavilay P, Muller CP, Black AP. *Lasting benefit of infant hepatitis B vaccination in adolescents in the Lao People's Democratic Republic*. International Journal of Infectious Diseases 2020 Jan. doi: https://doi.org/10.1016/j.ijid.2020.01.055

Hefele L, Syphan S, Xayavong D, Homsana A, Kleine D, Chanthavilay P, Nouanthong P, Xaydalasouk K, Phathammavong O, Billamay S, Xeuatvongsa A, Reinharz D, Black AP, Muller CP, 2020. *Timeliness of immunisation with the pentavalent vaccine at different levels of the health care system in the Lao People's Democratic Republic: A cross-sectional study.* PLoS One 1–15. https://doi.org/10.1371/journal.pone.0242502

b. Manuscripts in preparation

Hefele L, Black AP, Trinh Van T, Nyguyen Minh T, Nguyen Duc H, Virachith S, Muller CP, Hübschen J, Russell P, Bartholdson Scott J, Chau Nguyen Ngoc M, Tran Vu Thieu N, Baker S. *An age-stratified serosurvey against purified Salmonella enterica serovar Typhi antigens in the Lao People´s Democratic Republic.*

Hefele L, Black AP, Muller CP, Hübschen JM. *Haemophilus influenzae serotype B seroprevalence in Central provinces in Lao People´s Democratic Republic before and after vaccine introduction.*

Hefele L, Xaydalasouk K, Kleine D, Homsana A, Xayavong D, Sengdavanh S, Hübschen JM, Muller CP, Black AP. *Seroprevalence of measles and rubella antibodies in vaccinated and unvaccinated infants in the Lao People´s Democratic Republic.*

Hefele L, Nouanthong P, Black AP, Muller CP. *Hepatitis B virus infection in the Lao People's Democratic Republic: A systematic review.*

B. LIST OF PRESENTATIONS

Luxembourg Institute of Health - Department Meeting, Esch-sur-Alzette (Luxembourg) | Oral presentation | July 2020

"*Epidemiological and serological investigations of vaccine-preventable diseases and their implications for vaccination policy in the Lao People's Democratic Republic*"

ASVAC conference, Yangon (Myanmar) | Poster presentation | Sep 2019

"*Timeliness of the pentavalent childhood vaccine in selected health care facilities in Bolikhamxay province*"

ASVAC conference, Yangon (Myanmar) | Poster presentation | Sep 2019

"Lasting benefit of infant hepatitis B vaccination in adolescents in the Lao People's Democratic Republic."

ASVAC conference, Yangon (Myanmar) | Poster presentation | Sep 2019

"Seroprotection at different levels of the health care system after routine vaccination with DTPw-HepB-Hib in Lao PDR."

Vaccine congress, Bangkok (Thailand) | Poster presentation | Sep 2019

"*Timeliness of the pentavalent childhood vaccine in selected health care facilities in Bolikhamxay province*"

Vaccine congress, Bangkok (Thailand) | Oral presentation | Sep 2019

"Lasting benefit of infant hepatitis B vaccination in adolescents in the Lao People's Democratic Republic."

Luxembourg Institute of Health - Department Meeting, Esch-sur-Alzette (Luxembourg) | Oral presentation | Oct 2018

"*Seroprotection at different levels of the health care system after routine vaccination with DTPw-HepB-Hib in Lao PDR*"

Vaccine conference, Budapest (Hungary) | Poster presentation | Sep 2018

"Seroprotection at different levels of the health care system after routine vaccination with DTPw-HepB-Hib in Lao PDR"

PhD Days, Esch-sur-Alzette (Luxembourg) | Poster presentation | Nov 2018

"Seroprotection at different levels of the health care system after routine vaccination with DTPw-HepB-Hib in Lao PDR"

C. SUPPLEMENTARY MATERIAL OF THE RESEARCH MANUSCRIPTS

a. An age-stratified serosurvey measuring exposure to *Salmonella enterica* serovar Typhi in the Lao People´s Democratic Republic

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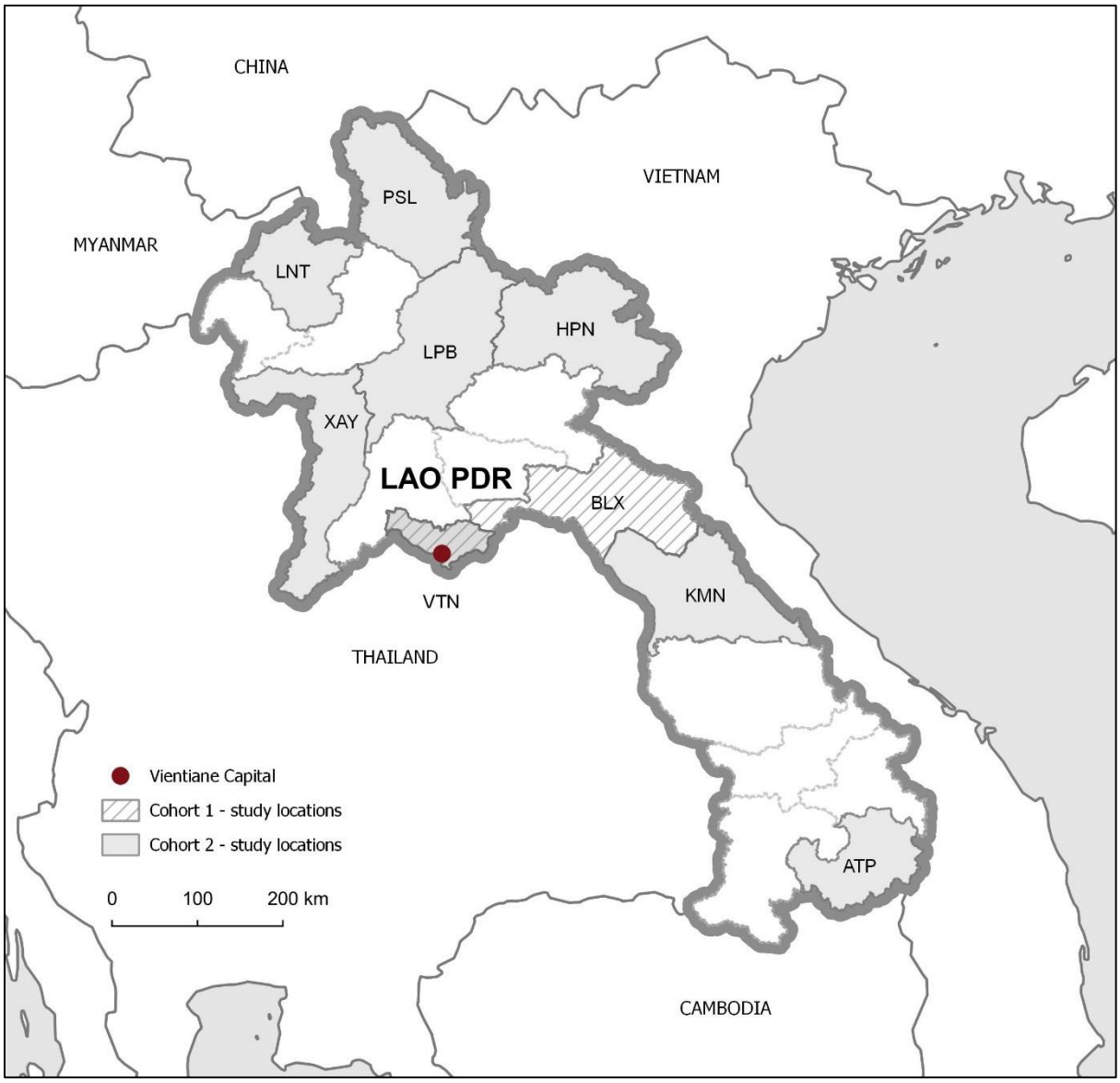
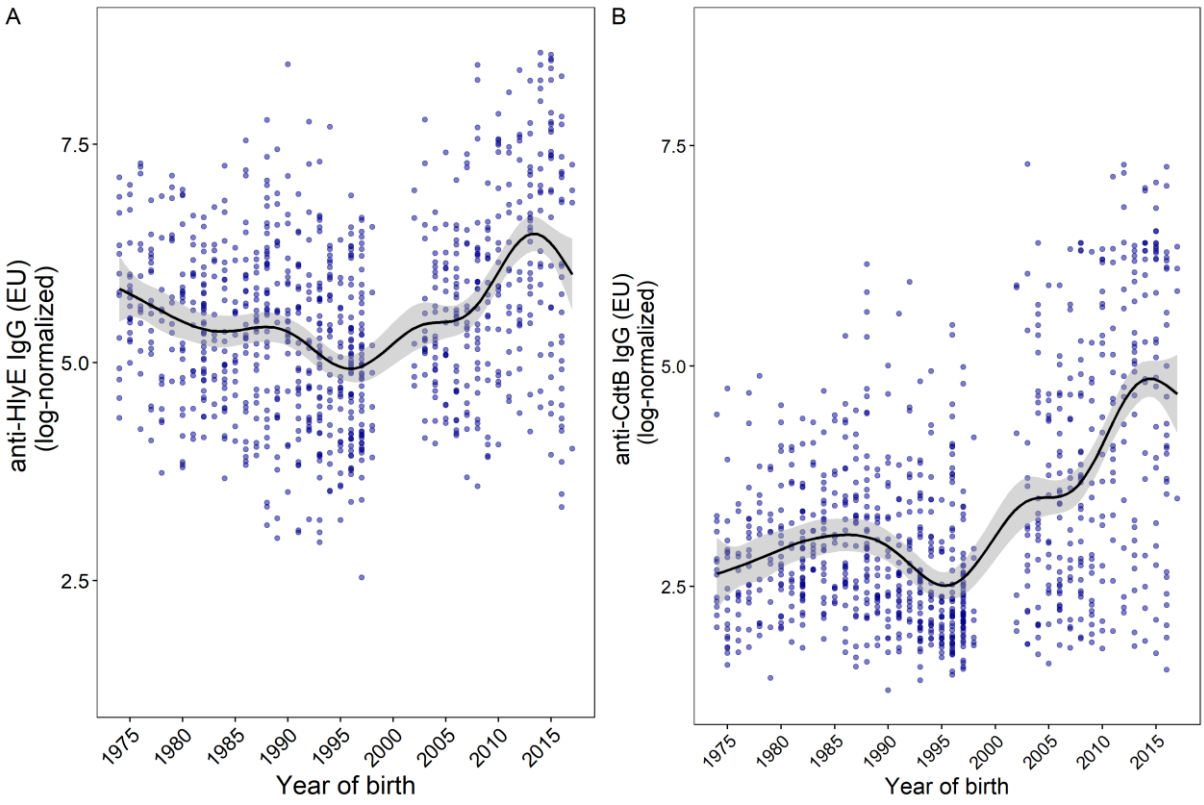
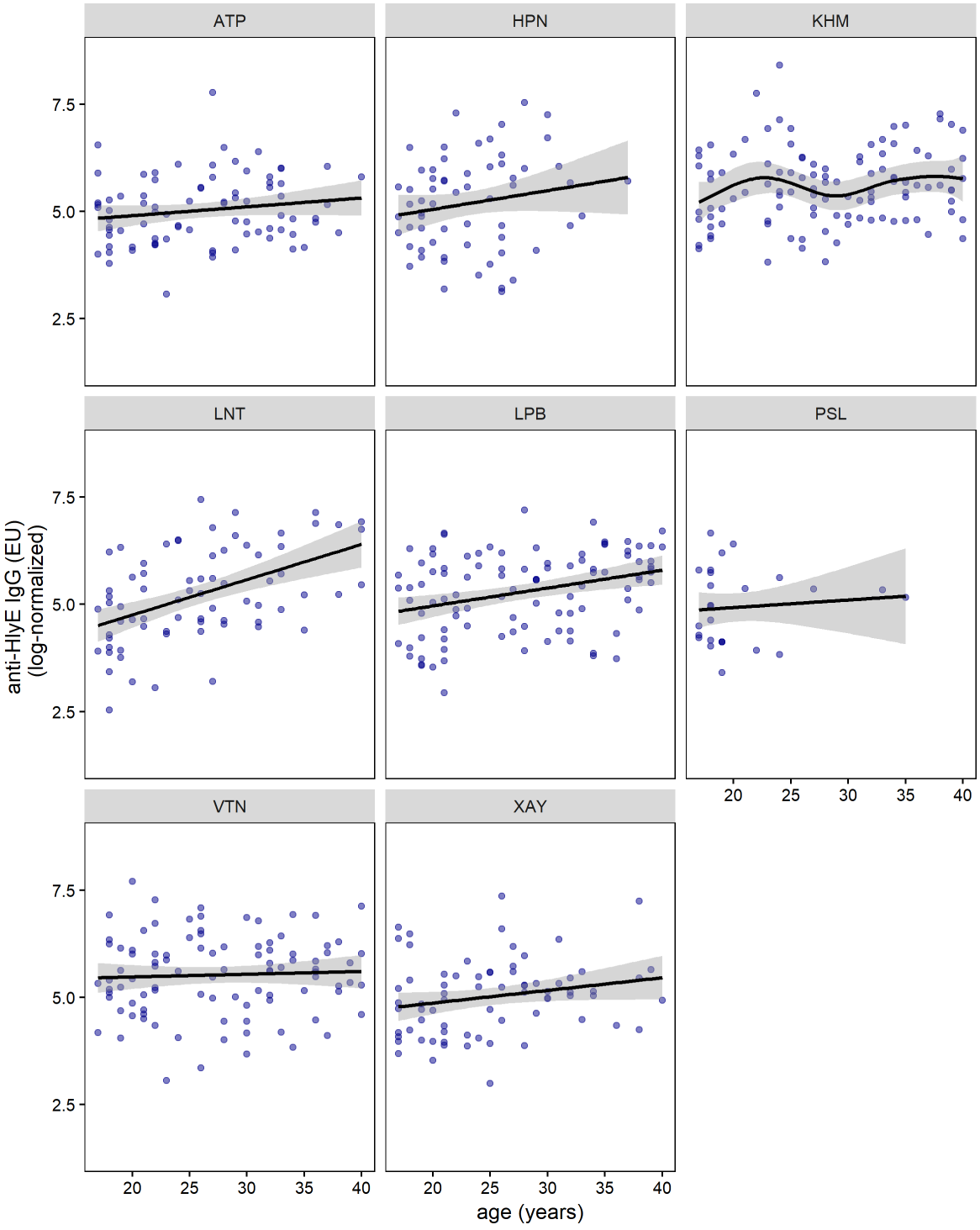
Supplementary material



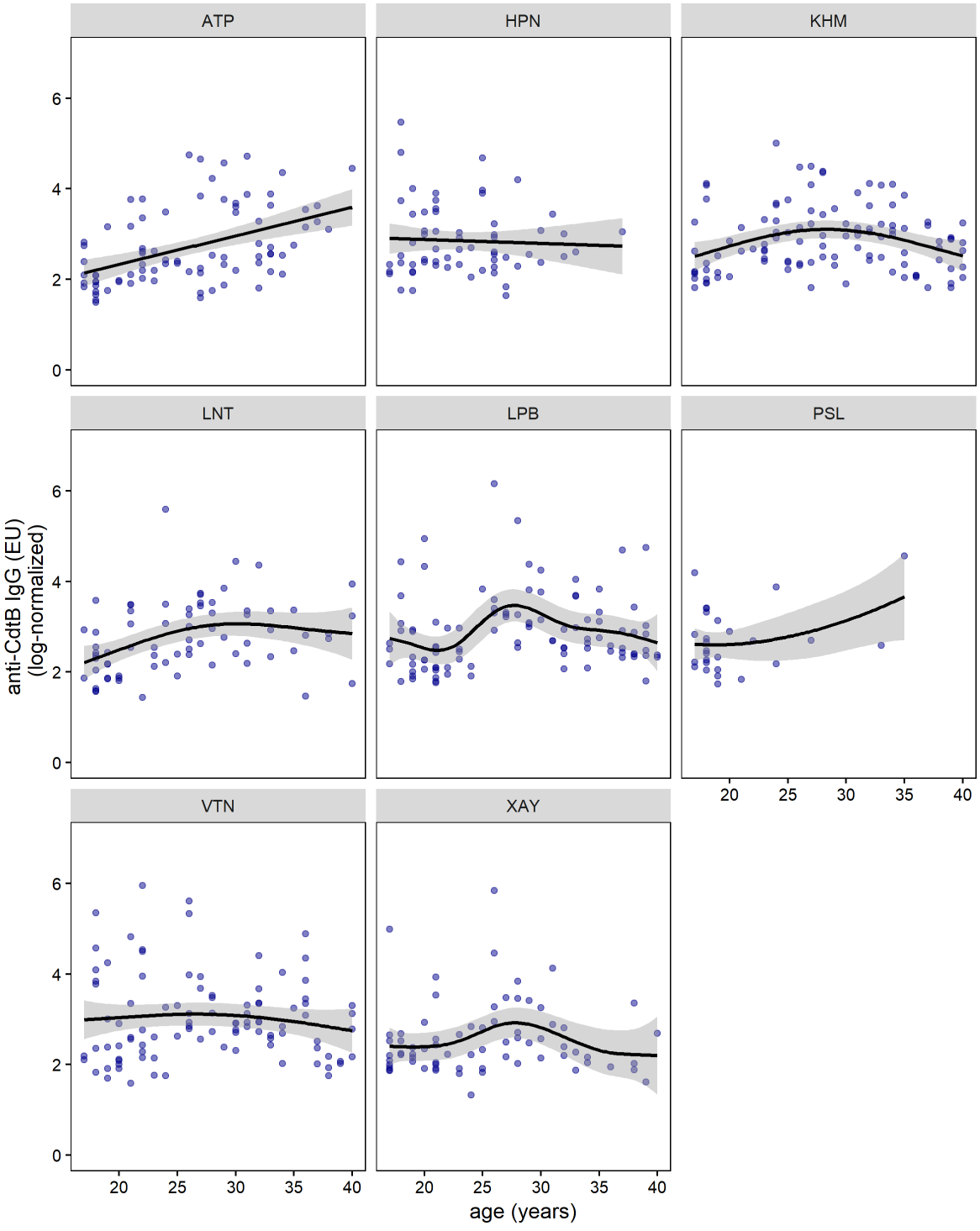
Supplementary Figure 1 Map of study sites generating serum samples for anti-S. Typhi IgG serology. The map was created with QGIS (QGIS Development Team, 2018) using the world borders dataset (http://thematicmapping.org/downloads/world_borders.php, 2019). The data regarding the administrative boundaries of Lao PDR was obtained from the Humanitarian Data Exchange website (https://data.humdata.org/dataset/lao-admin-boundaries, dataset provided by the National Geographic Department of Lao PDR, 2019). Projection used: EPSG 4326 – WGS 84. PSL = Phongsaly, LNT = Luang Namtha, HPN = Huaphan, LPB = Luang Prabang, XAY = Xayabouli, VTN = Vientiane, BLX = Bolikhamxay, KHM = Khammouane, ATP = Attapeu.



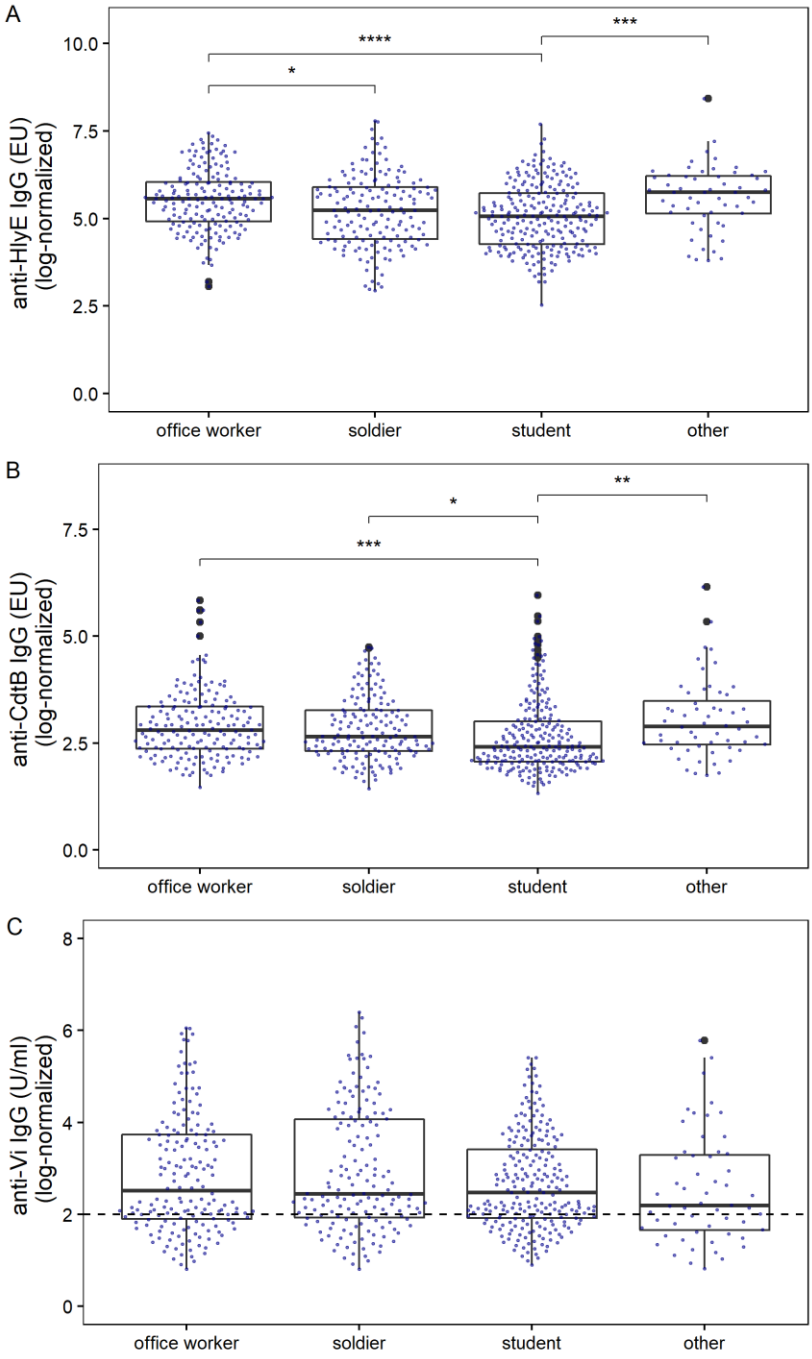
Supplementary Figure 2 Results of generalized additive and linear models assessing anti–S. Typhi IgG antibody prevalence in children and adults in Lao PDR as a function of birth year. Non-linear smooths were fitted for birth year in the model for anti-HlyE IgG (A) and anti-CdtB IgG (B) data. Shaded bands represent the pointwise 95%-confidence interval.



Supplementary Figure 3 Results of the generalized additive model assessing anti-HlyE IgG titer in adults in Lao PDR as a function of age by province. Shaded bands represent the pointwise 95%-confidence interval. ATP = Attapeu, HPN = Huaphan, KHM = Khammouane, LNT = Luang Namtha, LPB = Luang Prabang, VTN = Vientiane, PSL = Phongsaly, XAY = Xayabouli.



Supplementary Figure 4 Results of the generalized additive model looking assessing anti-CdtB IgG titer in adults in Lao PDR a function of age by province. Shaded bands represent the pointwise 95%-confidence interval. ATP = Attapeu, HPN = Huaphan, KHM = Khammouane, LNT = Luang Namtha, LPB = Luang Prabang, VTN = Vientiane, PSL = Phongsaly, XAY = Xayabouli.



Supplementary Figure 5 The distribution of anti–S. Typhi serum IgG titers in adults in Lao PDR by occupation. Each dot shows the measurement of an individual sample for (A) anti-HlyE IgG, (B) anti-CdtB IgG and (C) anti-Vi IgG with an underlying boxplot. Differences between groups were assessed using Kruskal-Wallis test followed by Dunn's *post-hoc* test with Bonferroni correction: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Participants whose occupation is not specified are grouped into "other". The dashed line in panel C represents the censoring limit, all data points below were treated as left-censored data.

b. *Haemophilus influenzae* serotype B seroprevalence in Central provinces in the Lao PDR before and after vaccine introduction

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Supplementary Material

Supplement Table 1 Anti-Hib IgG prevalence according to participants' characteristics for each study cohort

Predictor	Categories	n (% of total N)	Hib serology		
			<0.15 µg/ml	0.15-1.0 µg/ml	>1.0 µg/ml
All participants		1057 (100)	1.5	43.3	55.2
Unvaccinated adolescents (Cohort 1; N=296)					
All		296 (100)	4.1	50.3	45.6
Province	Vientiane Capital	148 (50.0)	2.0	48.0	50.0
	Bolikhamxay	148 (50.0)	6.1	52.7	41.2
District	Vientiane Capital	148 (50.0)	2.0	48.0	50.0
	Paksan	81 (27.4)	9.9	50.6	39.5
	Pakkading	67 (22.6)	1.5	55.2	43.3
Sex	Male	141 (47.6)	2.1	48.9	48.9
	Female	155 (52.4)	5.8	51.6	42.6
Age	<13 years	108 (36.5)	6.5	42.6	50.9
	13-16 years	108 (36.5)	1.9	54.6	43.5
	>16 years	80 (27.0)	3.8	55.0	41.3
Ethnicity	Tai Kadai	283 (95.6)	4.2	50.9	44.9
	Hmong-Mien & Mon-Khmer	13 (4.4)	0.0	38.5	61.5
Place of birth	Central Hospital in Vientiane	71 (24.0)	1.4	45.1	53.5
	Other & Unknown	225 (76.0)	4.9	52.0	43.1
N of household members	≤4	88 (29.7)	8.0	50.0	42.0
	>4	208 (70.3)	2.4	50.5	47.1
Vaccinated children (Cohort 2; N=761)					
All		761 (100)	0.5	40.6	58.9
Province	Vientiane Province	178 (23.4)	0.0	37.1	62.9
	Bolikhamxay	228 (30.0)	0.0	38.2	61.8
	Khammouane	355 (46.6)	1.1	43.9	54.9
Age	<1 year	45 (5.9)	0.0	20.0	80.0
	1-2 years	406 (53.4)	0.2	42.9	56.9

	>2 years	310 (40.7)	1.0	40.6	58.4
Sex	Male	381 (50.1)	0.5	40.9	58.5
	Female	380 (49.9)	0.5	40.3	59.2
Place of birth	Home	234 (30.7)	1.3	41.9	56.8
	Health Care Facility	524 (68.9)	0.2	39.9	59.9
	Unknown	3 (0.4)	0.0	66.7	33.3
Time since vaccination	<1 year	236 (31.0)	0.0	33.9	66.1
	1-2 years	378 (49.7)	0.5	43.9	55.6
	>2 years	133 (17.5)	1.5	45.1	53.4
	NA	14 (1.8)	0.0	21.4	78.6
Weight for height z-score	≥-2	684 (89.9)	0.6	40.1	59.4
	<-2	61 (8.0)	0.0	50.8	49.2
	Unknown	16 (2.1)	0.0	25.0	75.0
Height for age z-score	≥-2	430 (56.5)	0.5	39.1	60.5
	<-2	306 (40.2)	0.7	43.1	56.2
	Unknown	25 (3.3)	0.0	36.0	64.0
Weight for age z-score	≥-2	590 (77.5)	0.3	40.0	59.7
	<-2	166 (21.8)	1.2	42.8	56.0
	Unknown	5 (0.7)	0.0	40.0	60.0
Mid-upper arm circumference z-score	≥-2	719 (94.5)	0.6	39.6	59.8
	<-2	37 (4.9)	0.0	59.5	40.5
	Unknown	5 (0.7)	0.0	40.0	60.0
Parasite infection	No	383 (50.3)	0.3	40.5	59.3
	Yes	84 (11.0)	1.2	45.2	53.6
	Unknown	294 (38.6)	0.7	39.5	59.9

c. Seroprotection on different levels of the health care system after vaccination with DTPw-HepB-Hib in the Lao PDR

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Supplementary Material

1 **Supplementary data**

2 **Tables**

3 **Appendix Table A1** Socio-demographic characteristics of participants and parents/guardians
4 recruited at the Children's Hospital in Vientiane Capital and at different health care facilities
5 in Bolikhamxay province.

Variable	Categories	Children's Hospital (8 to 23 months)		Bolikhamxay (8 to 28 months)		Data from Bolikhamxay according to health care level					
		N=306		Total N = 819		PH N = 191		DH N = 184		HC N = 144	
		n	%	n	%	n	%	n	%	n	%
Accompanying person	Mother	297	97.1	660	80.6	158	82.7	145	78.8	357	80.4
	Father	6	2.0	57	7.0	12	6.3	13	7.1	32	7.2
	Other	3	1.0	102	12.5	21	11.0	26	14.1	55	12.4
Marital status of accompanying person	Unmarried	1	0.3	2	0.2	0	0.0	1	0.5	1	0.2
	Married	305	99.7	791	96.6	189	99.0	182	98.9	420	94.6
	Separated	0	0.0	3	0.4	0	0.0	1	0.5	2	0.5
	Divorced	0	0.0	23	2.8	2	1.0	0	0.0	21	4.7
	Not provided	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Age of the mother	< 20 years	1	0.3	58	7.1	1	0.5	13	7.1	44	9.9
	20 – 30 years	158	51.6	507	61.9	94	49.2	120	65.2	293	66.0
	> 30 years	147	48.0	185	22.6	70	36.6	27	14.7	88	19.8
	NA			69	8.4	26	13.6	24	13.0	19	4.3
Distance to nearest health care facility	< 10 km	258	84.3	591	72.2	140	73.3	170	92.4	281	63.3
	> 10 km	48	15.7	228	27.8	51	26.7	14	7.6	163	36.7
Travel time to HCF (dry season)	< 20 min	211	69.0	554	67.6	140	73.3	172	93.5	242	54.5
	20 - 40 min	88	28.8	214	26.1	51	26.7	12	6.5	151	34.0
	> 40 min	7	2.3	51	6.2	0	0.0	0	0.0	51	11.5
Travel time to HCF (rainy season)	< 20 min	196	64.1	465	56.8	101	52.9	157	85.3	207	46.6
	20 - 40 min	100	32.7	199	24.3	90	47.1	15	8.2	94	21.2
	> 40 min	10	3.3	155	18.9	0	0.0	12	6.5	143	32.2
Mode of transport to HCF	Private car or private Tuktuk	230	75.2	117	14.3	71	37.2	37	20.1	9	2.0
	Other peopls's car	0	0.0	9	1.1	0	0.0	2	1.1	7	1.6
	Bus or Tuktuk	1	0.3	6	0.7	0	0.0	5	2.7	1	0.2
	by foot	1	0.3	34	4.2	4	2.1	2	1.1	28	6.3
	Motorcycle	74	24.2	652	79.6	116	60.7	138	75.0	398	89.6
	Partic./Parents never go there (…)	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	Other	0	0.0	1	0.1	0	0.0	0	0.0	1	0.2
Ethnicity of parents / guardian	Mon-Khmer	0	0.0	36	4.4	1	0.5	7	3.8	28	6.3
	Tai-Kadai	299	97.7	676	82.5	190	99.5	165	89.7	321	72.3
	Hmong-Mien	3	1.0	107	13.1	0	0.0	12	6.5	95	21.4
	Other	4	1.3								
Occupation of mother	Housewife	83	27.1	239	29.2	89	46.6	69	37.5	81	18.2
	Labourer	4	1.3	14	1.7	6	3.1	4	2.2	4	0.9
	Farmer	0	0.0	389	47.5	24	12.6	59	32.1	306	68.9
	Trader	76	24.8	66	8.1	22	11.5	17	9.2	27	6.1
	Gov. employee	120	39.2	106	12.9	49	25.7	32	17.4	25	5.6
	Priv. employee	22	7.2	4	0.5	1	0.5	2	1.1	1	0.2
	Other	1	0.3	1	0.1	0	0.0	1	0.5	0	0.0
Monthly income of household	< 500,000 Kip	0	0.0	128	15.6	12	6.3	12	6.5	104	23.4
	500,000 – 1,000,000 Kip	3	1.0	211	25.8	20	10.5	50	27.2	141	31.8
	1,000,000 – 2,000,000 Kip	49	16.0	336	41.0	108	56.5	80	43.5	148	33.3
	> 2,000,000 Kip	249	81.4	139	17.0	50	26.2	42	22.8	47	10.6
	Not provided / unknown	5	1.6	5	0.6	1	0.5	0	0.0	4	0.9
Completed level of education of mother	None	0	0.0	70	8.5	3	1.6	12	6.5	55	12.4

1

	Primary School	7	2.3	330	40.3	40	20.9	55	29.9	235	52.9
	Secondary School	139	45.4	292	35.7	73	38.2	83	45.1	136	30.6
	Prof. training / College	82	26.8	75	9.2	41	21.5	22	12.0	12	2.7
	University	78	25.5	52	6.3	34	17.8	12	6.5	6	1.4
Receive Antenatal Care	No	1	0.3	72	8.8	2	1.0	16	8.7	54	12.2
	Yes	304	99.3	742	90.6	187	97.9	167	90.8	388	87.4
	Unknown	1	0.3	5	0.6	2	1.0	1	0.5	2	0.5
Age of child	≤ 12 months	186	60.8	201	24.5	46	24.1	41	22.3	114	25.7
	> 12 months	120	39.2	618	75.5	145	75.9	143	77.7	330	74.3
Gender of the child	Male	156	51.0	433	52.9	91	47.6	90	48.9	252	56.8
	Female	150	49.0	386	47.1	100	52.4	94	51.1	192	43.2
Place of birth	Home	0	0.0	149	18.2	7	3.7	25	13.6	117	26.4
	Health center	0	0.0	167	20.4	0	0.0	1	0.5	166	37.4
	District hospital	0	0.0	259	31.6	3	1.6	134	72.8	122	27.5
	Provincial hospital	8	2.6	201	24.5	158	82.7	16	8.7	27	6.1
	Central hospital	298	97.4	24	2.9	11	5.8	7	3.8	6	1.4
	Other	0	0.0	19	2.3	12	6.3	1	0.5	6	1.4
Exclusive breast-feeding	Yes	270	88.2	72	8.8	167	87.4	159	86.4	421	94.8
	No	36	11.8	747	91.2	24	12.6	25	13.6	23	5.2
Duration exclusive breast-feeding	< 6 months	130	42.5	552	67.4	148	77.5	118	64.1	286	64.4
	≥ 6 months	176	57.5	267	32.6	43	22.5	66	35.9	158	35.6
Age of addition of rice to diet	< 6 months	8	2.6	309	37.7	80	41.9	68	37.0	161	36.3
	≥ 6 months	298	97.4	510	62.3	111	58.1	116	63.0	283	63.7
Number of siblings	< 2	153	50.0	266	32.5	64	33.5	61	33.2	141	31.8
	≥ 2	153	50.0	553	67.5	127	66.5	123	66.8	303	68.2
Number of household members	< 6	300	98.0	432	52.7	123	64.4	97	52.7	212	47.7
	≥ 6	6	2.0	387	47.3	68	35.6	87	47.3	232	52.3

6

2

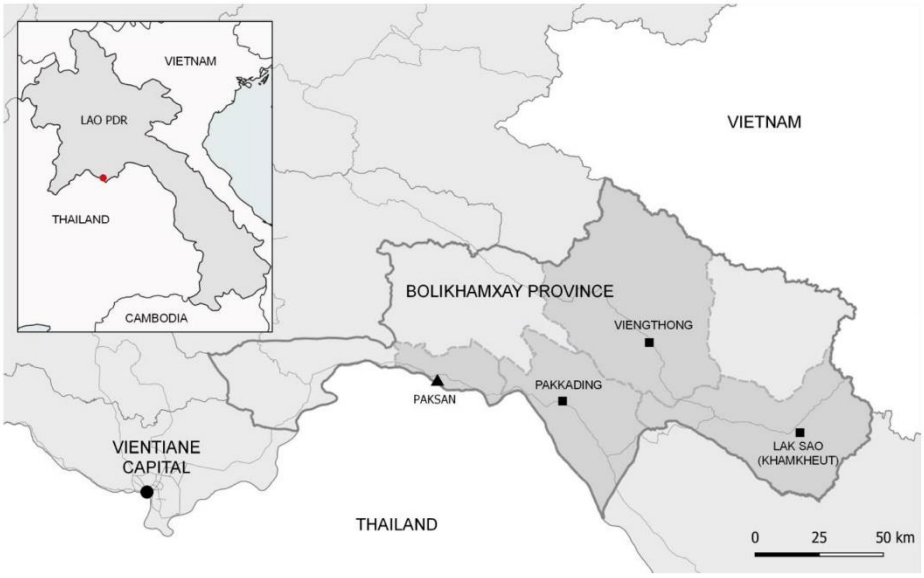
Appendix Table A2 Bivariate analyses: Data were dichotomized into participants with anti-Diphtheria IgG greater or less than 0.1 IU/ml, anti-tetanus IgG greater or less than 0.5 IU/ml, anti-HBs IgG greater or less than 100 IU/L, anti-Hib IgG greater or less than 1.0 µg/ml and anti-Pertussis greater or less than 22 IU/ml.

		anti-diphtheria				anti-tetanus				anti-HBs				anti-Hib				anti-pertussis			
Variable	**Categories (N)[1]**	**% protected**	**OR**	**95% CI**	**p-value**	**% protected**	**OR**	**95% CI**	**p-value**	**% protected**	**OR**	**95% CI**	**p-value**	**% protected**	**OR**	**95% CI**	**p-value**	**% protected**	**OR**	**95% CI**	**p-value**
Socio-economic factors																					
Occupation of mother	Housewife (219-220)	85.39	1.00			80.00	1.00			48.64	1.00			74.43			NS	27.40			NS
	Labourer + Farmer (374-378)	71.16	0.42	[0.27-0.65]	**<0.0001**	71.43	0.63	[0.42-0.93]	**0.0249**	41.16	0.74	[0.53-1.03]	**0.0876**	69.79				23.20			
	Trader + Employee + Other (161-163)	81.71	0.76	[0.44-1.32]	0.4004	78.05	0.89	[0.54-1.46]	0.7035	40.49	0.72	[0.48-1.08]	**0.1203**	76.40				24.54			
Level of education of mother	None (68)	66.18	1.00			61.76	1.00			41.18			NS	57.35	1.00			20.59			NS
	Primary school (300-306)	77.56	1.77	[1.00-3.12]	**0.0607**	75.49	1.91	[1.10-3.32]	**0.0241**	46.56				71.33	1.85	[1.08-3.18]	**0.0297**	24.83			
	Secondary school + University (386-390)	79.49	1.98	[1.13-3.46]	**0.0185**	77.58	2.14	[1.24-3.69]	**0.009**	40.87				76.17	2.28	[1.39-4.06]	**0.0026**	25.32			
Household income	< 500,000 Kip (121-125)	65.85	1.00			65.85	1.00			40.80			NS	63.64	1.00			28.69			NS
	500,000 -1,000,000 Kip (199)	83.42	2.61	[1.54-4.42]	**0.0004**	77.39	1.77	[1.08-2.92]	**0.0281**	45.23				71.86	1.46	[0.90-2.36]	**0.1365**	23.12			
	> 1,000,000 Kip (434-440)	78.13	1.85	[1.20-2.87]	**0.0064**	77.05	1.74	[1.13-2.69]	**0.0139**	42.92				75.35	1.75	[1.14-2.68]	**0.0149**	24.31			
Travel time to nearest HCF (rainy season)	< 20 min (428-431)	79.44	1.00			77.73	1.00			42.59			NS	74.30			NS	23.44			NS
	20 - 40 min (183-186)	82.26	1.20	[0.77-1.87]	0.4418	78.92	1.07	[0.70-1.63]	0.8318	45.41				71.04				27.92			
	> 40 min (143-147)	65.99	0.50	[0.33-0.76]	**0.0016**	63.70	0.50	[0.33-0.76]	**0.0014**	41.50				69.23				25.00			
Travel time to nearest HCF (dry season)	< 20 min (510-513)	79.84	1.00			77.58	1.00			43.66			NS	74.31	1.00			24.19			NS
	20 - 40 min (197-200)	78.50	0.92	[0.62-1.38]	**0.6810**	76.50	0.94	[0.64-1.39]	0.7659	44.22				72.59	0.92	[0.63-1.33]	0.6346	24.59			
	> 40 min (49-50)	50.00	0.25	[0.14-0.46]	**<0.0000**	46.94	0.26	[0.14-0.46]	**<0.0000**	34.00				53.19	0.39	[0.21-0.72]	**0.0033**	26.39			
Distance to nearest HCF	< 10 km (543-547)	78.90	1.00			76.78	1.00			43.69			NS	74.77	1.00			25.32			NS
	>10 km (211-216)	71.07	0.76	[0.53-1.10]	**0.1501**	71.63	0.76	[0.53-1.09]	**0.1612**	41.86				66.82	0.68	[0.48-0.96]	**0.0296**	23.11			
Vaccinee related factors																					
Ethnicity of parents / guardians	Tai-Kadai (619-624)	83.01	1.00			77.92	1.00			44.55	1.00			76.09	1.00			25.65			NS
	Hmong-Mien + Mon-Khmer (135-138)	52.55	0.23	[0.15-0.34]	**<0.0001**	63.50	0.49	[0.33-0.73]	**0.0006**	36.96	0.73	[0.50-1.07]	**0.1074**	56.30	0.40	[0.27-0.60]	**<0.0001**	20.44			
Age of participant	≤ 12 months (187-189)	83.60	1.00			88.36	1.00			57.22	1.00			79.68	1.00			29.26	1.00		
	>12 months (572-575)	75.52	0.61	[0.39-0.93]	**0.0209**	71.03	0.32	[0.20-0.52]	**<0.0001**	38.61	0.47	[0.34-0.66]	**<0.0001**	70.19	0.60	[0.40-0.90]	**0.0138**	23.20	0.73	[0.50-1.06]	**0.0982**
Place of birth	Home (141-144)	75.00	1.00			76.92	1.00		NS	39.58			NS	70.92			NS	23.78	1.00		
	Health center + District hospital (391-397)	74.87	0.99	[0.64-1.54]	1.0000	72.47	0.79			44.08				71.36				21.94	0.90	[0.57-1.42]	0.6414
	Provincial & Central hospital + Other (221-223)	83.86	1.75	[1.03-2.91]	**0.0435**	79.37	0.87			43.89				75.68				30.18	1.39	[0.86-2.24]	**0.1898**
Duration exclusive breastfeeding	< 6 months (512-516)	82.91	1.00			77.91	1.00			44.19			NS	74.80	1.00			23.05			**NS**
	≥ 6 months (242-246)	66.26	0.40	[0.29-0.57]	**<0.0001**	69.92	0.66	[0.47-0.93]	**0.0194**	41.06				67.77	0.71	[0.51-0.99]	**0.0449**	25.49			
Vaccine related factors																					
District	Paksan (175)	82.86	1.00			81.25	1.00			45.71			NS	74.86	1.00			32.00	1.00		
	Khamkheut & Viengthong (420-428)	72.24	0.54	[0.34-0.84]	**0.0066**	72.54	0.59	[0.38-0.93]	**0.0219**	40.42				69.52	0.77	[0.51-1.14]	**0.1987**	22.93	0.63	[0.43-0.93]	**0.0235**
	Pakkading (159-761)	77.53	1.24	[0.69-2.24]	0.5496	75.78	0.70	[0.41-1.18]	0.2285	47.80				77.99	1.19	[0.72-1.98]	0.5216	21.38	0.58	[0.35-0.95]	**0.0356**
Location of vaccination: Groups																					
All levels	Provincial hospital (176)	82.95	1.00			81.25	1.00			45.45			NS	74.43			NS	31.82	1.00		
	District hospital 8176-178)	84.09	1.08	[0.62-1.91]	0.8859	75.54	0.72	[0.43-1.21]	0.2446	42.13				72.00				15.34	0.39	[0.23-0.65]	**0.0004**
	Health center (403-409)	72.37	0.54	[0.34-0.84]	**0.0063**	72.55	0.61	[0.39-0.94]	**0.0283**	42.65				71.96				25.68	0.74	[0.50-1.09]	**0.1309**
All levels divided in outreach service and vaccination at facility	Provincial hospital (176)	82.95	1.00			81.25			NS	45.45			NS	74.43	1.00			31.82	1.00		
	District hospital – at facility (113-114)	80.70	0.86	[0.47-1.58]	0.6409	75.44				42.98				78.76	1.27	[0.72-2.24]	0.4799	16.81	0.43	[0.24-0.78]	**0.0057**
	District hospital – by outreach service (62-64)	90.32	1.92	[0.76-4.86]	0.2166	76.56				40.63				59.68	0.51	[0.28-0.94]	**0.0351**	12.70	0.31	[0.14-0.70]	**0.0028**
	Health center – at facility (90-91)	82.42	0.96	[0.49-1.88]	1.0000	75.82				43.96				74.44	1.00	[0.56-1.79]	1.0000	31.11	0.97	[0.56-1.67]	1.0000
	Health center – by outreach service (313-318)	69.50	0.47	[0.30-0.74]	**0.0012**	71.61				42.27				71.25	0.85	[0.56-1.29]	0.4629	24.13	0.68	[0.45-1.03]	**0.0716**
Health centers grouped acc. to travel time to district hospital	Provincial hospital (176)	82.95	1.00			81.25	1.00			45.45	1.00			74.43	1.00			31.82	1.00		
	District hospital (175-178)	84.09	1.09	[0.62-1.91]	0.8859	75.44	0.72	[0.43-1.21]	0.2446	42.13	**0.87**	[0.57-1.33]	0.5923	72.00	0.88	[0.55-1.42]	0.6312	15.34	0.39	[0.23-0.65]	**0.0004**
	< 30 min (166-168)	86.90	1.36	[0.75-2.47]	0.3607	80.84	0.97	[0.57-1.67]	1.0000	50.00	1.20	[0.78-1.84]	0.4485	78.92	1.29	[0.78-2.13]	0.3716	24.70	0.70	[0.44-1.13]	**0.1517**
	30 to 60 min (126-128)	73.23	0.56	[0.32-0.98]	**0.0463**	80.47	0.95	[0.53-1.69]	0.8833	47.66	1.09	[0.69-1.72]	0.7278	77.78	1.20	[0.70-2.06]	0.5859	22.83	0.63	[0.38-1.07]	**0.0933**
	> 60 min (111-114)	50.00	0.21	[0.12-0.35]	**<0.0001**	51.33	0.24	[0.14-0.41]	**<0.0001**	26.32	0.43	[0.26-0.71]	**0.0012**	54.95	0.42	[0.25-0.69]	**0.0008**	30.36	0.93	[0.56-1.56]	0.8963

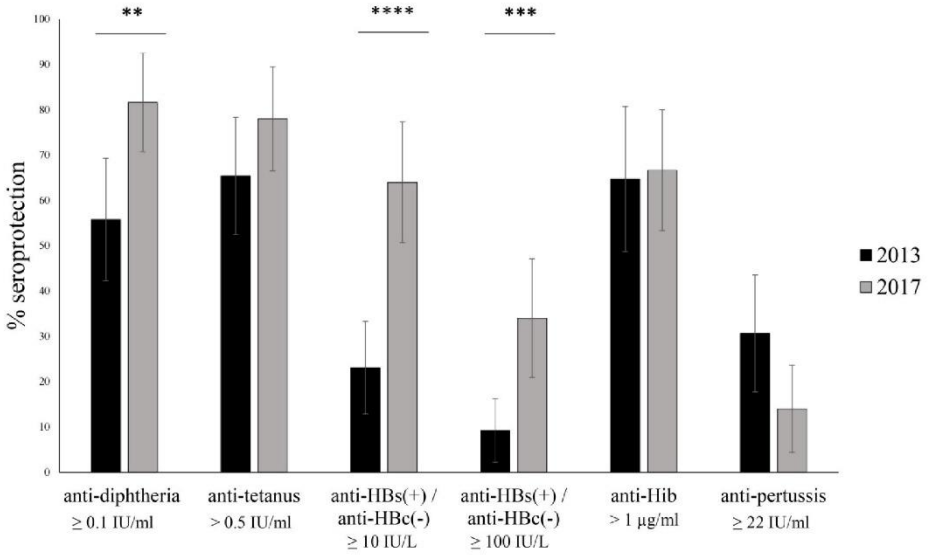
[1]total numbers for the categories vary between the analyses of the vaccine components; we could not list them individually due to space limitations

Variables including seroconversion to individual components that were not significant for at least two of the vaccine antigens are not shown in the table: Number of siblings, Number of household members, Age of mother, Gender of child, Exclusive breastfeeding, Rice in diet, ANC, Tetanus vaccination during pregnancy, Number of Tetanus vaccination during pregnancy and Hepatitis B birth dose

Appendix Figure A1 Map of Lao PDR and location of study sites: Vientiane Capital (black circle), Paksan (capital city of Bolikhamxay province, black triangle), Pakkading, Viengthong and Lak Sao (capital cities of Pakkading, Viengthong and Khamkheut district, black square). The travel time by car from Vientiane Capital to Paksan is approximately 3 hours, the travel time from Paksan to either the district capital of Khamkheut or Viengthong is 4-5 hours and the travel time from Paksan to Pakkading is approximately 1 hour. The map was created with QGIS (QGIS Development Team, 2018) using collected GPS-data and the world borders dataset (http://thematicmapping.org/downloads/world_borders.php, 2019). The data regarding the administrative boundaries of Lao PDR was obtained from the Humanitarian Data Exchange website (https://data.humdata.org/dataset/lao-admin-boundaries, dataset provided by the National Geographic Department of Lao PDR, 2019). Projection used: EPSG 4326 – WGS 84.



Appendix Figure A2 Comparison of the serological profiles of children aged 8 to 28 months enrolled in Viengthong district in the same villages in 2017 and 2013/14. Serpoprotection rates are displayed with 95% confidence intervals. ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.



d. Seroprevalence of measles and rubella antibodies in vaccinated and unvaccinated infants in the Lao People´s Democratic Republic

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Supplementary Materials

Supplementary Table 1 Participants characteristics by recruitment site

Variable	Categories	Children's Hospital (8 to 23 months) N=288		Bolikhamxay (8 to 28 months) N=802	
		n	%	n	%
Socio-economic factors					
Accompanying person	Mother	281	97.57	647	80.67
	Father	4	1.39	57	7.11
	Other	3	1.04	98	12.22
Marital status of accompanying person	Unmarried	1	0.35	2	0.25
	Married	287	99.65	775	96.63
	Separated	0	0.00	3	0.37
	Divorced	0	0.00	22	2.74
Age of the mother	< 20 years	1	0.35	57	7.11
	20 – 30 years	145	50.35	495	61.72
	> 30 years	142	49.31	183	22.82
	NA		0.00	67	8.35
Distance to nearest HCF	≤ 10km	243	84.38	580	72.32
	> 10 km	45	15.63	222	27.68
Travel time to HCF (dry season)	≤ 20 min	237	82.29	643	80.17
	> 20 min	51	17.71	159	19.83
Travel time to HCF (rainy season)	≤ 20 min	226	78.47	565	70.45
	> 20 min	62	21.53	237	29.55
Mode of transport to HCF	Private car or private Tuktuk	221	76.74	116	14.46
	Other peopls's car	0	0.00	9	1.12
	Bus or Tuktuk	1	0.35	6	0.75
	by foot	1	0.35	34	4.24
	Motorcycle	65	22.57	636	79.30
	Other	0	0.00	1	0.12
Ethnicity of parents / guardian	Mon-Khmer	0	0.00	33	4.11
	Tai-Kadai	281	97.57	664	82.79

	Hmong-Mien	3	1.04	105	13.09
	Other	4	1.39		0.00
Occupation of mother	Housewife	77	26.74	238	29.68
	Labourer	4	1.39	14	1.75
	Farmer	0	0.00	380	47.38
	Trader	73	25.35	62	7.73
	Gov, employee	115	39.93	103	12.84
	Priv, employee	18	6.25	4	0.50
	Other	1	0.35	1	0.12
Monthly income of household	< 500,000 Kip	0	0.00	122	15.21
	500,000 – 1,000,000 Kip	3	1.04	211	26.31
	1,000,000 – 2,000,000 Kip	46	15.97	328	40.90
	> 2,000,000 Kip	234	81.25	136	16.96
	Not provided / unknown	5	1.74	5	0.62
Completed level of education of mother	None	0	0.00	68	8.48
	Primary School	7	2.43	323	40.27
	Secondary School	130	45.14	285	35.54
	Prof, training / College	79	27.43	74	9.23
	University	72	25.00	52	6.48
Receive Antenatal Care	No	1	0.35	72	8.98
	Yes	286	99.31	725	90.40
	Unknown	1	0.35	5	0.62
Number of household members	< 6	282	97.92	423	52.74
	≥ 6	6	2.08	379	47.26
Vaccinee-related factors					
Age of child	≤ 12 months	172	59.72	195	24.31
	> 12 months	116	40.28	607	75.69
Gender of the child	Male	149	51.74	426	53.12
	Female	139	48.26	376	46.88
Place of birth	Home	0	0.00	143	17.83
	Health center	0	0.00	165	20.57
	District hospital	0	0.00	253	31.55
	Provincial hospital	8	2.78	198	24.69
	Central hospital	280	97.22	19	2.37
	Other	0	0.00	24	2.99
Exclusive breast-feeding	Yes	256	88.89	70	8.73
	No	32	11.11	732	91.27
Duration exclusive breast-feeding	< 6 months	122	42.36	542	67.58
	≥ 6 months	166	57.64	260	32.42
Age of addition of rice to diet	< 6 months	8	2.78	305	38.03
	≥ 6 months	280	97.22	497	61.97
Number of siblings	< 2	145	50.35	261	32.54
	≥ 2	143	49.65	541	67.46
Vaccine-related factors					
Vaccinated with MR	yes	120	41.67	652	81.30
	no (incl. those vaccinated at day of recruitment)	168	58.33	133	16.58
	Status unknown	0	0.00	17	2.12
Recruitment site	CH	288	100.00	0	
	PH	na		190	23.69
	DH	na		177	22.07
	HC	na		435	54.24
		CH (n = 120)		*BLX (n = 652)*	
if vaccinated, place of vaccination	CH	120	100.00	0	0.00
	PH			160	24.39
	DH			154	23.48
	HC			338	51.52

Supplementary Table 2 Age at vaccination by health care level

Health care level	Age at vaccination[1] in months		
	mean	median	min-max
Vientiane Capital			
CH	10	10	9 - 23
Bolikhamxay			
All	11	10	3 - 21
PH	10	10	3 - 17
DH - facility	11	10	5 - 20
DH - outreach	11	11	9 - 15
HC - facility	11	11	9 - 18
HC - outreach	11	11	6 - 21
NA	10	10	10 - 10

[1]Participants for which the calculated time value was negative were removed, since it indicates a mistake made in the vaccination records.

CH = Central Hospital, PH = Provincial Hospital, DH = District Hospital, HC = Health Center, NA = place of vaccination outside scope of study.

e. Timeliness of immunisation with the pentavalent vaccine at different levels of the health care system in the Lao People's Democratic Republic: a cross-sectional study

Timeliness of immunisation with the pentavalent vaccine at different levels of the health care system in the Lao People's Democratic Republic: a cross-sectional study

Short title Delayed vaccination in Lao PDR

Authors

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Supporting information

Participants

1174 participants were originally enrolled in the context of an immunogenicity study (Hefele et al., 2019). For this study, 12 participants were excluded from the 1174 participants who were recruited originally, because they were vaccinated at a health care facility outside of the scope of this study or because the vaccination history could not be verified conclusively. In the immunogenicity study, 49 participants were excluded due to the same reasons and because it was not possible to obtain a serum sample or because the serum sample was not sufficient for the laboratory analyses.

Tables

S1 Table. Range of the difference between two vaccination dates in days according to health care level and dose.

		Difference between mismatches[1]			
	Health care level	mean	median	min	max
Pentavalent 1	PH	1.41	0	0	70
	DH - facility	6.72	0	-33	315
	DH - outreach	-2.32	0	-92	0
	HC - facility	7.46	0	-26	372
	HC - outreach	-2.23	0	-366	272
Pentavalent 2	PH	0.99	0	0	65
	DH - facility	-3.11	0	-273	77
	DH - outreach	1.58	0	-3	33
	HC - facility	-2.88	0	-111	41
	HC - outreach	-6.08	0	-192	77
Pentavalent 3	PH	1.16	0	-31	62
	DH - facility	-0.02	0	-268	113
	DH - outreach	0.55	0	-91	85
	HC - facility	-9.92	0	-165	42
	HC - outreach	-9.34	0	-482	330

[1]Participants whose birthdates did not match in the records were excluded
PH = Provincial hospital, DH = District hospitals, HC = Health centers

S2 Table. Impact of receiving the pentavalent dose 3 later than 16 weeks on seroconversion.

Place of vaccination	Antibody status		n age at pentavalent 3 > 16 weeks / N per group (%)	OR	95% CI	p-value
Central hospitals in Vientiane Capital	anti-Tetanus IgG	not protected	24/44 (54.5)	ref	-	NS
	(N tested = 299)	protected (>0.5 IU/ml)	159/255 (62.4)	0.72	[0.38-1.38]	
	anti-Diphtheria IgG	not protected	12/23 (52.2)	ref	-	NS
	(N tested = 301)	protected (≥0.1 IU/ml)	172/278 (61.9)	0.67	[0.29-1.58]	
	anti-HBs IgG	not protected	21/38 (55.3)	ref	-	NS
	(N tested = 302)	protected (≥10 IU/ml)	164/264 (62.1)	0.75	[0.38-1.5]	
	anti-*Haemophilus influenzae* type b IgG	not protected	2/2 (100)	ref	-	NS
	(N tested = 301)	protected (>1.0 IU/ml)	182/299 (60.9)	NA	NA	
	anti-Pertussis IgG	not protected	108/189 (57.1)	ref	-	NS
	(N tested = 301)	protected (>22 IU/ml)	76/112 (67.9)	0.63	[0.39-1.03]	
Provincial hospital in Bolikhamxay	anti-Tetanus IgG	not protected	18/33 (54.5)	ref	-	NS
	(N tested = 181)	protected (>0.5 IU/ml)	106/148 (71.6)	0.48	[0.22-1.03]	
	anti-Diphtheria IgG	not protected	18/29 (62.1)	ref	-	NS
	(N tested = 181)	protected (≥0.1 IU/ml)	106/152 (69.7)	0.71	[0.31-1.62]	
	anti-HBs IgG	not protected	22/37 (59.5)	ref	-	NS
	(N tested = 181)	protected (≥10 IU/ml)	102/144 (70.8)	0.60	[0.29-1.23]	

anti-*Haemophilus influenzae* type b IgG	not protected	33/49 (67.3)	ref	-	NS
(N tested = 181)	protected (>1.0 IU/ml)	91/132 (68.9)	0.93	[0.46-1.87]	
anti-Pertussis IgG	not protected	85/123 (69.1)	ref	-	NS
(N tested = 181)	protected (>22 IU/ml)	39/58 (67.2)	1.1	[0.56-2.13]	

CI = Confidence interval, NS = not significant; NA = not available

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47 S3 Table. Median interval in weeks between doses according to health care level.

	Interval between vaccinations[1]							
	Pentavalent 1 to 2				Pentavalent 2 to 3			
Health care level	N	Median (IQR, in weeks)	4 weeks %	>5 weeks %	N	Median (IQR, in weeks)	4 weeks %	>5 weeks %
CH	319	4.71 (4.43-5.00)	64.89	33.86	319	4.57 (4.43-5.00)	69.28	30.41
PH	197	4.57 (4.43-5.00)	64.97	29.95	196	4.57 (4.43-5.04)	62.24	30.1
DH - facility	127	5.00 (4.86-5.86)	23.62	72.44	125	5.00 (4.96-6.00)	23.2	74.4
DH - outreach	68	5.14 (4.57-8.07)	30.88	64.71	69	5.21 (4.64-9.82)	26.09	68.12
HC - facility	97	4.71 (4.43-6.29)	52.58	42.27	93	4.71 (4.43-8.04)	52.69	45.16
HC - outreach	333	5.36 (4.43-8.82)	36.94	57.96	333	6.00 (4.43-8.86)	37.04	63.06
Total	1141	4.86 (4.43-5.86)	49.08	47.06	1135	4.86 (4.43-6.14)	48.63	48.28

[1]The age was calculated with the date written in the vaccination card, if the vaccination card was not present, the date in the hospital records was used; dates that were unreadable, did not exist or where only signature was present, were not included in this table. Children vaccinated at different health care levels were not included.
IQR = Interquartile range, CH = Central hospitals, PH = Provincial hospital, DH = District hospitals, HC = Health centers

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50 S4 Table. Participants grouped according to timing of intervals between vaccinations with the pentavalent
51 vaccine.

	CH	PH	DH - facility	DH - outreach	HC - facility	HC - outreach	Mix
Total N	319	196	119	65	91	330	42
both intervals 4 weeks (%)	50.47	47.96	10.92	10.77	38.46	19.39	26.19
at least 1 interval longer than 4 weeks (%)	48.28	42.35	83.19	80.00	57.14	73.03	66.67
Mixed (%)	1.25	9.69	5.88	9.23	4.40	7.58	7.14

CH = Central hospitals, PH = Provincial hospital, DH = District hospitals, HC = Health centers, N = total numbers of participants vaccinated at the respective health care level

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54 S5 Table. Factors associated with timely completion of primary vaccination with the pentavalent vaccine (in
55 Bolikhamxay province) by 24 weeks.

Variables		n completed/N per group (%)[1]	OR	95% CI	p-value
Socio-economic Factors					
Occupation of mother	Labourer + Farmer+ Trader + Employee + Other	191/241 (79.25)	1.00		
	Housewives	423/592 (71.45)	0.66	[0.46-0.94]	0.024
Mother's level of education (completed)	None + primary education level	256/405 (63.21)	1.00		
	Secondary school + University	358/428 (83.64)	2.98	[2.15-4.12]	<0.001
Household income (month)	< 1.000.000 Kip	212/344 (61.63)			
	> 1.000.000 Kip	402/489 (82.21)	2.88	[2.09-3.95]	<0.001
Number of siblings	< 2	205/267 (76.78)	1.00		
	≥ 2	409/566 (72.26)	0.79	[0.56-1.11]	0.178
Number of household members	< 6	329/432 (76.16)	1.00		
	≥ 6	285/401 (71.07)	0.77	[0.56-1.05]	0.099
Travel time to nearest HCF	< 20 min	461/580 (79.48)	1.00		
	> 20 min	153/253 (60.47)	0.39	[0.29-0.55]	<0.001
Distance to nearest HCF[2]	< 10 km	451/608 (74.18)			NS
	>10 km	163/225 (72.44)			
Vaccinee related factors					
Ethnicity of parents / guardians	Tai-Kadai	539/681 (79.15)	1.00		
	Hmong-Mien + Mon-Khmer	75/152 (49.34)	0.26	[0.18-0.37]	<0.001
Age of mother	≤ 30 years	363/522 (69.54)	1.00		
	> 30 years	199/243 (81.89)	1.98	[1.36-2.89]	<0.001
Age of participant	≤ 12 months	145/209 (69.38)	1.00		
	>12 months	469/624 (75.16)	1.34	[0.95-1.89]	0.103
Gender of participant	Male	324/436 (74.31)			NS
	Female	290/397 (73.05)			
Place of birth[3]	Home	87/155 (56.13)	1.00		
	Health center + District hospital	308/436 (70.64)	1.88	[1.29-2.75]	0.001
	Provincial + Central hospital + Other	219/242 (90.5)	7.44	[4.36-12.7]	<0.001
Exclusive breastfeeding	No	54/73 (73.97)			NS
	Yes	560/760 (73.68)			
Duration exclusive breastfeeding	< 6 months	416/557 (74.69)			NS
	≥ 6 months	198/276 (71.74)			
Received antenatal care[4]	No + I do not know	41/80 (51.25)	1.00		
	Yes	573/753 (76.1)	3.03	[1.89-4.84]	<0.001
TT vaccination during ANC[4]	No + I do not know	87/147 (59.18)	1.00		
	Yes	527/686 (76.82)	2.29	[1.57-3.32]	<0.001
Number of tetanus doses during ANC[4]	0 -3	209/326 (64.11)	1.00		
	4-5	405/507 (79.88)	2.22	[1.62-3.04]	<0.001
Hepatitis B birth dose	Yes	515/640 (80.47)	1.00		
	No	99/193 (51.3)	0.26	[0.18-0.36]	<0.001
Vaccine related factors					
District	Paksan	173/187 (92.51)	1.00		
	Khamkheut & Viengthong	288/464 (62.07)	0.13	[0.07-0.24]	<0.001
	Pakkading	153/182 (84.07)	0.43	[0.22-0.84]	0.014
Place of vaccination	Vaccinated at Health care facility	349/384 (90.89)	1.00		
	Vaccinated in Outreach	224/394 (56.85)	0.13	[0.09-0.2]	<0.001

[1] The date in the vaccination card was used to calculated the age at pentavalent 3. In case the vaccination card was not available, the hospital recrods were used

[2] Distance and travel time to nearest HCF contained similar information, only the variable travel time was included in multivariable analyses

[3] Place of birth and district correlated with place of vaccination, the variables was not included in multivariable analyses

[4] From those variables regarding ANC services, only the main variable of having had ANC or not was included in multivariable analyses

CI = Confidence interval, NS = not significant; HCF = health care facility; ANC = antenatal care

S6 Table. The timeliness of receiving the hepatitis B birth dose by health care level.

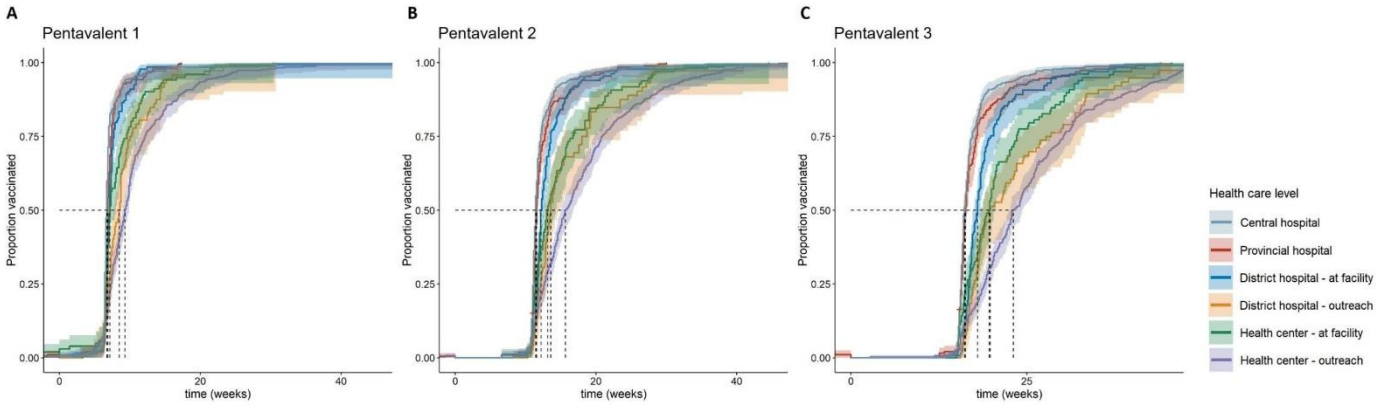
	Health care level							
	CH	PH	DH - facility	DH – outreach	HC - facility	HC - outreach	total	NA[1]
N received birth dose[2]	270	187	206	2	132	24	821	57
on birth date (%)	93.0	97.9	93.2	100.0	92.4	75.0	93.5	80.7
within 1 week (%)	5.9	1.1	4.4	0.0	3.0	12.5	4.1	19.3
After 7 days (%)	1.1	1.1	2.4	0.0	4.5	12.5	2.3	0.0

[1]NAs = participants born outside study, dates not readable, missing, or only in month and year, parents did not know place of vaccination

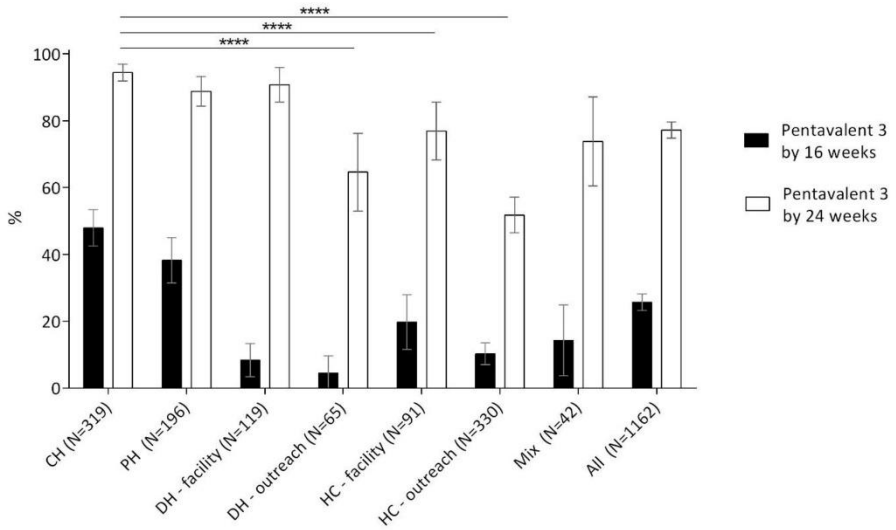
[2]Total number does not contain participants whose birth dates did not match in the records, or whose time difference between birth and vaccination was negative

CH = Central hospitals, PH = Provincial hospital, DH = District hospitals, HC = Health centers

Figures

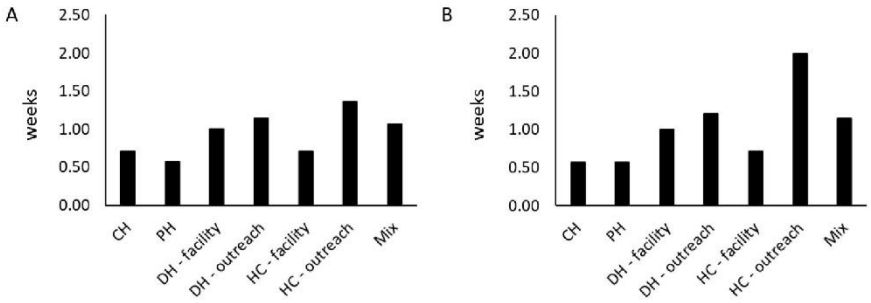


S1 Fig. Timeliness of each dose of the pentavalent vaccine according to the age and the health care levels. A. Timeliness of vaccination with pentavalent 1. B. Timeliness of vaccination with pentavalent 2. C. Timeliness of vaccination with pentavalent 3. Shaded areas indicate the 95% Confidence Interval. Graphs were truncated at 45 weeks to increase visibility. CH = vaccinated at central Hospitals in Vientiane, PH = vaccinated at provincial hospital, DH = vaccinated at district hospital level, HC = vaccinated at health center level. Dashed lines correspond to the median age of vaccination with the pentavalent vaccine. Participants for which the calculated age at vaccination was negative (date of dose before date of birth, indicating a mistake in documentation) were excluded from the graph.



S2 Fig. Proportion of participants vaccinated with the third dose of the pentavalent vaccine by health care levels. Mix = participants vaccinated at different health care facilities with one or two of the doses. CH = Central hospital level, DH = District hospital level, HC = health center level. Missing or unreadable dates were excluded from this figure. The age at vaccination was calculated based on the vaccination card, and in case the vaccination card was not available the date in the hospital record was used. The proportion vaccinated at PH, the DH and HC level was compared to the CH level. Data are presented with 95% CI. **** = p<0.0001.

81



82

83 S3 Fig. Difference of interval as recommended in schedule and calculated median interval between pentavalent
84 dose 1 and 2 (A) and pentavalent dose 2 and 3 (B) in weeks according to health care level. The intervals were
85 calculated based on the vaccination cards, and in case the vaccination card was not available the date in the hospital
86 records was used. CH = Central hospital level, DH = District hospital level, HC = health center level. Missing or
87 unreadable dates were excluded from this figure.

88

f. Lasting benefit of infant hepatitis B vaccination in adolescents in the Lao PDR

Lasting benefit of infant hepatitis B vaccination in adolescents in the Lao People's Democratic Republic

Running title Hepatitis B markers in Lao adolescents

Authors

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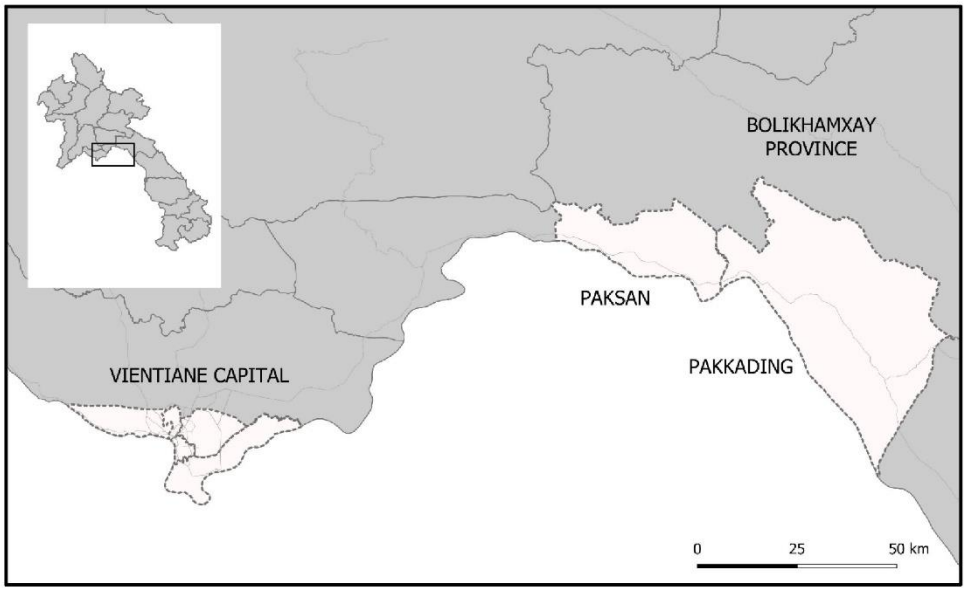
16 **SUPPLEMENTARY DATA**

17 **Supplementary Table 1.** Characteristics of the participants in Vientiane Capital and Bolikhamxay province

		Total		Vientiane Capital		Bolikhamxay	
Variable	**Categories**	**N = 779**	**%**	**N=388**	**%**	**N=391**	**%**
Location of school							
	PKD highschool (Pakkading district)	100	12.84			100	25.58
	Viengkham (Pakkading district)	94	12.07			94	24.04
	Sithanasai (Paksan district)	69	8.86			69	17.65
	Pak Niep (Paksan district)	66	8.47			66	16.88
	Nongbua (Paksan district)	62	7.96			62	15.86
	Chao Anouvong Highschool (Vientiane Capital)	98	12.58	98	25.26		
	Vientiane Highschool (Vientiane Capital)	147	18.87	147	37.89		
	Hongkha Highschool (Vientiane Capital)	46	5.91	46	11.86		
	Bo Na Ngua Highschool (Vientiane Capital)	97	12.45	97	25.00		
Parents (living situation)							
	Single parent	46	5.91	37	9.54	9	2.30
	Both Father and Mother	623	79.97	299	77.06	324	82.86
	Other	110	14.12	52	13.40	58	14.83
Gender							
	Male	380	48.78	182	46.91	198	50.64
	Female	399	51.22	206	53.09	193	49.36
Age of participants (years)							
	11	56	7.19	26	6.70	30	7.67
	12	84	10.78	35	9.02	49	12.53
	13	95	12.20	49	12.63	46	11.76
	14	121	15.53	59	15.21	62	15.86
	15	104	13.35	47	12.11	57	14.58
	16	105	13.48	58	14.95	47	12.02
	17	93	11.94	54	13.92	39	9.97
	18	121	15.53	60	15.46	61	15.60
Place of birth							
	Central Hospital in Vientiane Capital	196	25.16	185	47.68	11	2.81
	Provincial Hospital	78	10.01	15	3.87	63	16.11
	District Hospital	31	3.98	5	1.29	26	6.65
	Health Center	27	3.47	0	0.00	27	6.91
	Home	178	22.85	29	7.47	149	38.11
	Other	18	2.31	7	1.80	11	2.81
	I do not know	251	32.22	147	37.89	104	26.60
Ethnicity							
	Lao Loum (Tai-Kadai)	745	95.64	382	98.45	363	92.84
	Lao Soung (Hmong-Mien)	5	0.64	1	0.26	4	1.02
	Lao Theung (Mon-Khmer)	14	1.80	4	1.03	10	2.56

	I do not know	644		317		327	
Mode of transportation to the nearest health care facility							
	By foot	16	2.05	2	0.52	14	3.58
	Motorbike	423	54.30	172	44.33	251	64.19
	Private car	336	43.13	211	54.38	125	31.97
	Bicycle	2	0.26	1	0.26	1	0.26
	Other	2	0.26	2	0.52	0	0.00



Supplementary Figure 1 Map of study sites in Lao PDR. Districts of Vientiane Capital and Paksan and Pakkading district in Bolikhamxay Province are shaded in light grey. The map was created with QGIS (QGIS Development Team, 2018) using collected GPS-data and the world borders dataset (http://thematicmapping.org/downloads/world_borders.php, 2019). The data regarding the administrative boundaries of Lao PDR was obtained from the Humanitarian Data Exchange website (https://data.humdata.org/dataset/lao-admin-boundaries, dataset provided by the National Geographic Department of Lao PDR, 2019). Projection used: EPSG 4326 – WGS 84.

D. POLICY BRIEFS

a. Health policy brief: Disparity in the response to routine immunisation with the pentavalent vaccine at different levels of the health care system

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Health Policy Brief

Disparity in the response to routine immunisation with the pentavalent vaccine at different levels of the health care system

Executive abstract
Children in remote areas are less likely to have an adequate vaccine response after vaccination with the full course of DTP-HepB-Hib vaccine. We suggest a booster vaccination especially in remote areas.

1. Background

Lao People's Democratic Republic (PDR) has made remarkable progress to reduce the burden of vaccine-preventable infectious diseases over the last 20 years. However, vaccination coverage remains challenging and is compounded by sometimes low vaccine immunogenicity in the Lao setting. The country has a high adult prevalence of chronic hepatitis B virus infections and there are outbreaks of vaccine preventable infectious diseases in all age groups[1–4]. The pentavalent vaccine against diphtheria, tetanus, pertussis, hepatitis B and *haemophilus influenzae* type b is scheduled as part of the Expanded Programme of Immunization at 6, 10 and 14 weeks of age[5]. We aimed to determine the immune response to vaccination (immunogenicity) in Lao children with documented three doses of the vaccine given at different levels of the health care system.

2. Research findings

A first study regarding the vaccine immunogenicity of the pentavalent childhood vaccination was conducted in 2013/14 in Bolikhamxay, Khammouane and Vientiane province[6] and a second one in 2017 in Bolikhamxay province. Seroprotective rates were as low as 38.47% for Hepatitis B, 54.32% against Tetanus, 56.89% against Diphtheria and 58.85% against *Haemophilus influenzae* type b in 2013/14 (Figure 1). In 2017, the responses to the vaccine had dramatically improved and were mostly above 70%. When the 2017 data was grouped according to the place of vaccination and the age groups, the response differed little between the Children's Hospital, a Central Hospital in Vientiane, the Provincial Hospital in Bolikhamxay, and the District Hospitals in Viengthong, Lak Sao and Pakkading. However, protection was still suboptimal particularly in remote settings when health centers were grouped according to the travel time to the nearest district hospital (Figure 2). Children that were vaccinated by more remote health centres were less likely to be protected against Diphtheria, Tetanus and Hepatitis B. Therefore, in addition to low vaccine coverage, relatively low response to vaccination remains a concern at least in remote parts of Lao PDR. Our findings suggest that many children are still at risk despite a full course of vaccination and protection rates tend to be lower in more distant and hard-to-reach settings. The limitations of

these conclusions are that they are based on only selected districts in the Bolikhamxay province and only children with documented three vaccinations were enrolled and therefore any flaws in documentation may appear as low vaccine response.

3. Policy

In order to address the lower levels of seroprotection in hard-to-reach settings, we recommend to review and strengthen vaccine management with a focus on the "last-mile" of the supply chain regarding administration, the training of the staff, transportation and equipment.
In addition, the introduction of an additional booster dose at 18 months of age (at the time of the second measles/rubella vaccination) in particular in remote settings should be considered. A booster dose of the pentavalent vaccine could help to fill the gaps in coverage (catch-up) and may also boost immunity in those already vaccinated, counteracting the waning of protective antibodies.

4. Impact

Children in remote areas are less well covered by vaccination and in general they may be more likely to be exposed to vaccine preventable diseases and have more difficult access to health care. Our study suggests that are also more likely have a lower response to the vaccine. This requires further attention since 71% of the population live in rural areas and an estimated 70% of the children are vaccinated through outreach services [7,8], which are primarily focusing on populations in remote areas.
A recent study suggests that poor villages should be targeted to improve coverage rates in Lao PDR [5]. It has been shown that vaccines are frequently exposed to temperatures outside the recommended range after they left the provincial storage facilities[9]. Thus, a focus on remote areas would improve the coverage and management issues that our study and others have identified.
By including an additional vaccine of DTP-Hep-Hib into the Lao routine schedule particularly for this vulnerable population, it would act as a catch-up for those that were missed earlier, and boost those that received the early doses.
The pentavalent vaccine from Serum Institute India (currently used in Lao PDR) has been shown to be safe and immunogenic in 15-18-month olds [10]. Booster vaccinations against tetanus, diphtheria and pertussis as part of the immunization schedule are successful in prolonging the duration of protection. Neighbouring Asian countries have adopted DTP booster vaccination doses in their immunization schedule. Thailand includes a 4th and 5th dose of DTP at the age of 1.5 and 4 years [11] and the 4th dose of DTP is part of the schedule in Vietnam as well [12]. Hepatitis B booster doses do not seem to be part of the current booster vaccinations in either Thailand or Vietnam. Present vaccination recommendations do not consider booster doses to be necessary; however, the discussion is still ongoing in respect to country specific circumstances [13]. The inclusion of Hepatitis B in the form of the pentavalent vaccine could have an additional benefit given the high levels of chronic infection in Lao PDR [1] and the associated risk for children. Furthermore, low levels of pertussis protection (unpublished study) and reports of diphtheria outbreaks [2,14] emphasise the importance of boosting coverage and immunity to these diseases.

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b. Health policy brief: Seroprevalence of measles and rubella antibodies in vaccinated and unvaccinated infants in the Lao People´s Democratic Republic

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Seroprevalence of measles and rubella antibodies in vaccinated and unvaccinated infants in the Lao People´s Democratic Republic

1. Background

Even though measles vaccination was introduced in the Lao PDR in 1984, coverage rates remain consistently low and outbreaks continue to occur frequently. The measles vaccine is given in combination with rubella, which was added in 2011 [1–4]. In a recent study, the seroprevalence of measles and rubella antibodies in 1090 vaccinated and unvaccinated children from Vientiane Capital and Bolikhamxay province was investigated.

2. Research findings

Among vaccinated participants from Vientiane, 67.5% were positive/borderline for both measles and rubella antibodies (Table 1). An additional 29 (24.2%) were positive/borderline for only rubella and 1 (0.8%) was positive/borderline for only measles antibodies. The majority (75.6%) of the unvaccinated participants were double negative for both antibodies, but 24.4% were positive for anti-rubella and/or anti-measles IgG.

In Bolikhamxay only 498 (76.4%) of the vaccinated children were positive/borderline for both anti-measles and anti-rubella IgG and 19.2% were only positive/borderline for anti-rubella IgG (Table 1). Among the unvaccinated participants, 82 (61.7%) were double negative. A high proportion were double positive/borderline (25.6%) or only single positive for rubella IgG (12%) or measles IgG (0.8%). In logistic regression, having more than two siblings, a mother who is a farmer or labourer and having received the vaccine less than 180 days ago were independently associated with being less likely to be protected against measles/rubella in Bolikhamxay. When the proportion of vaccinated children from Bolikhamxay was compared to data collected during a previous study, the seroprevalence for anti-rubella IgG increased from 82% to 95.5% and anti-measles IgG increased from 66% to 76.4%.

The timeliness of the measles-rubella vaccination was also assessed: The median age was similar at both study sites (10 months in Vientiane Capital; 10-11 months in Bolikhamxay). However, the proportion of participants vaccinated after 12 months of age increased from 6% at the Central Hospitals to 33.7% - 46.7% in lower ranked health care facilities.

3. Impact and policy recommendation

In the past years, major progress has been made in the Lao PDR in vaccination coverage and seroprotection rates, but the National Immunization Programme still faces many challenges. Conducting serological studies to evaluate and monitor the vaccination programmes is important for identifying gaps in population immunity and impact of vaccination. Data on seroprevalence of MR vaccination in the Lao PDR are scarce and measles outbreaks occur frequently [3,5].

Our study shows a high prevalence of both anti-measles and anti-rubella antibodies in unvaccinated children, suggesting wide-spread circulation of the viruses and possibly underreporting of cases. Only 67.5% and 76.4% of vaccinated children were double positive/borderline for measles and rubella IgG

in Vientiane and Bolikhamxay province respectively. Seroprevalence in Bolikhamxay has improved but remains insufficient for establishing herd-immunity. At both study sites, more vaccinated children were single positive for rubella than for measles IgG, suggesting wider spread circulation of the rubella virus and/or higher immunogenicity of the rubella component of the vaccine.

We have previously observed vaccination delays on lower levels of the health care system for the pentavalent vaccine [6]. Vaccination coverage and vaccination timeliness are separate issues but they are connected: Delays in routine vaccination increase the risk of missed opportunities. Delayed vaccination also increases the window of disease susceptibility facilitating disease outbreaks.

RECOMMENDATIONS:

1. To conduct a thorough evaluation of vaccine management in the Lao PDR
2. To strengthen the surveillance of rubella and measles cases by systematically using the case definition for identifying suspected cases
3. To set-up systematic laboratory testing for improved reporting
4. To monitor timely completion of vaccinations

Table 1 Seroprevalence of measles and rubella IgG antibodies in vaccinated and unvaccinated children by recruitment site (participants with unclear vaccination status were not included; N=17)

				Rubella IgG positive & borderline n(%)	negative n(%)	total
VTN	vaccinated (N=120)	Measles IgG	positive & borderline n(%)	81 (67.5)	1 (0.8)	82 (68.3)
			negative n(%)	29 (24.2)	9 (7.5)	38 (31.7)
			total	110 (91.7)	10 (8.3)	**120**
	unvaccinated (N=168)	Measles IgG	positive & borderline n(%)	15 (8.9)	17 (10.1)	32 (19.0)
			negative n(%)	9 (5.4)	127 (75.6)	136 (81.0)
			total	24 (14.3)	144 (85.7)	**168**
BLX	vaccinated (N=652)	Measles IgG	positive & borderline n(%)	498 (76.4)	1 (0.2)	499 (76.5)
			negative n(%)	125 (19.2)	28 (4.3)	153 (23.5)
			total	623 (95.6)	29 (4.5)	**652**
	unvaccinated (N=133)	Measles IgG	positive & borderline n(%)	34 (25.6)	1 (0.8)	35 (26.3)
			negative n(%)	16 (12.0)	82 (61.7)	98 (73.7)
			total	50 (37.6)	83 (62.4)	**133**

VTN = Vientiane Capital; BLX = Bolikhamxay

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c. Health policy brief: Timeliness of immunisation with DTPw-HepB-Hib at different levels of the health care system in the Lao People's Democratic Republic

ສະຖາບັນ ປັດສະເຕີ ລາວ
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RESEARCH DEDICATED TO LIFE

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Health Policy Brief

Timeliness of immunisation with DTPw-HepB-Hib at different levels of the health care system in the Lao People's Democratic Republic

1. Background

The timely administration of vaccines is considered important for both coverage and vaccine response. Delayed vaccination of children may increase the risk for infection before vaccination, compromising the health of the vaccinees as well as herd immunity[1–3]. We investigated the timeliness of immunization with the diphtheria-tetanus-whole cell pertussis-hepatitis B-*Haemophilus influenzae* type b vaccine (DTPw-HepB-Hib), which is scheduled at 6, 10 and 14 weeks of age, in children with documented vaccination in Vientiane Capital and Bolikhamxay province in Lao PDR.

2. Research findings

1162 children aged 8-28 months who had received the full course of DTPw-HepB-Hib at different levels of the health care system were enrolled[4]. Vaccination dates documented in hospital records (HR) and/or yellow immunisation cards (YC) were both recorded for the purpose of the study.
In Bolikhamxay, both HR and YC were available for 56.8% of the participants. Discrepancies between both vaccination documents were observed. 38% of the participants had at least one mismatch among the three vaccination date-pairs. The proportion of vaccination date mismatches increased from dose 1 to dose 3 and with lower ranking of the health care facility (2.4% for dose 1 to 6% for dose 3 at the Provincial hospital (PH), 25.4% to 31.7% at the District hospitals (DH), 11.1% to 13% at the DH in outreach, 19% to 41.1% at the Health centers (HC) and 19.1% to 25.6% at the HC in outreach). At the Central hospital (CH) in Vientiane, we were able to review both HR and YC for 27.6% of the participants and all vaccination dates were in agreement.
In general, vaccination with DTPw-HepB-Hib was found to be delayed, especially vaccination with the last of the three doses and in health care settings below the PH level. The median delay of vaccination with dose 1 at the CH and PH level was 1 week while it was 3 weeks delay at the HC level for outreach vaccination. For dose 3, the median delay of vaccination was 2 weeks at the CH and PH level as compared to almost 9 weeks at the HC level for outreach vaccination).

Compared to the CH level, significantly less participants that were vaccinated at the HC and DH level received the third dose by 16 (48% at the CH vs. 7.1% at the DH and 12.4% at the HC) and 24 weeks of age (94.4% at the CH vs 62% at the DH-outreach and 57.2% at the HC) respectively, the maximal window recommended by WHO guidelines. Almost all participants (93.5%) in the study that received the hepatitis B birth dose did receive it on the day of their birth.

We used completion with DTPw-HepB-Hib 3 at 24 weeks as binary outcome to determine factors associated with timely completion of vaccination. In bivariate analyses, being an older mother with a higher education, a better job and higher household income was associated with the timely completion of vaccination. Other indicators of inequality in access to health care (i.e. delivery in peripheral health facilities or at home, no antenatal care and no hepatitis B birth dose for the participant) were significant

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d. Health policy brief: Impact of hepatitis B vaccination

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Health Policy Brief: Impact of hepatitis B vaccination

1. Background

Hepatitis B is endemic in Lao PDR with 8-10% of the adult population being chronically infected[1]. The introduction of HBV vaccines into the national immunization schedule in Lao PDR was started in 2001, with three doses of HBV vaccination at 6, 10 and 14 weeks. An additional dose given at birth (HBV birth dose) was introduced in 2003[1–3]. We investigated the impact of HBV vaccination on prevalence and correlates of HBV infection in adolescents born shortly before and after the vaccine introduction.

2. Research findings

779 students from Vientiane Capital and Bolikhamxay province were tested for HBV markers by ELISA. The proportion of students with a serological vaccination profile increased significantly between those born before and after the introduction of the vaccine in 2001 in Vientiane (13.2% to 21.9% ($p<0.05$)) and in Bolikhamxay (3.0% to 19.7% ($p<0.001$)), which translated to at least a two-times lower prevalence of past HBV infection (anti-HBc). The prevalence of HBsAg decreased slightly in Bolikhamxay but not in Vientiane, but the study may not have been sensitive enough to confirm this. Students in Bolikhamxay who were born earlier, i.e. older students, were less likely to be vaccinated and more likely to have been exposed to HBV.

This study showed a considerable reduction of past HBV infection in adolescents born after the introduction of the infant HBV vaccination. However, the proportion of students without any serological HBV markers ranged from 60% to 90% in those age groups, which is likely due to low vaccine coverage in the early years of vaccine introduction or to low vaccine immunogenicity.

3. Policy and impact

Our study identifies an immunity gap against hepatitis B in adolescents who were born between 2000 and 2007. To cover this gap these birth cohorts should be offered an additional dose of HBV vaccine. The most cost-effective way may be a school-based vaccination campaign. In case Lao PDR plans to introduce HPV vaccination in teenage girls, the HBV vaccine could be given at the same time point. Currently, hepatitis B vaccination is only offered at birth and at 6, 10 and 14 weeks of age by the National Immunization Programme in Lao PDR. In general, three doses of hepatitis B vaccination are recommended for unvaccinated individuals. Vaccination of unvaccinated adolescents may also follow an alternative schedule with two doses [4,5].

A study by Hutton et al. (2010)[6] investigated the cost-effectiveness of a hepatitis B catch-up vaccination for children and adolescents in China. They concluded that a free catch-up vaccination programme would be not only cost-saving but also have a significant health impact. Indeed, HBV infection rates in China were dramatically reduced by 2014 due to a combination of infant and catch-up vaccination programmes and a programme for preventing mother-to-child transmission.

In addition, awareness campaigns regarding the risk factors of HBV infection should be conducted in high schools. Even among Lao medical students [7] and health care workers [8], vaccination rates and knowledge are low. Furthermore, in order to prevent mother-to-child transmission of HBV, routine infant vaccination, especially of the birth dose, must be further strengthened. Although we observe a significant decrease in past HBV infection in those born post vaccination, the prevalence of chronic infection only decreased slightly in Bolikhamxay. This observation may indicate that the vaccination was successful in preventing infection later in life, but may have been unable to prevent transmission before 6 weeks of age. There is therefore an urgent need to increase the birth dose coverage above the 55% reported in 2017[9].

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E. CURRICULUM VITAE

Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht (mit Ausnahme der Publikationsliste).

PUBLICATIONS

Hefele, L., Kleine, D., Chanthavilay, P., Nouanthong, P., Xaydalasouk, K., Phathammavong, O., Billamay, S., Xeuatvongsa, A., Reinharz, D., Black, A.P., Muller, C.P., 2020. *Timeliness of immunisation with the pentavalent vaccine at different levels of the health care system in the Lao People' s Democratic Republic: A cross-sectional study*. PLoS One 1–15. https://doi.org/10.1371/journal.pone.0242502

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