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project

Establishing the linkages between foodborne bacterial enteropathies and malnutrition in Timor-Leste

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2 Executive summary

There is growing evidence of an association between infectious gastroenteritis and malnutrition in low-income countries (1-5). Enteric bacterial infections contracted in the crucial first years of life have been shown in several studies in low- and middle-income countries to damage the intestines and provoke a chronic inflammatory response leading to malabsorption of nutrients.

Drivers of enteropathogen infection in infants include zoonotic, environmental, and socio-economic factors, and thus the response must span human and animal health. A One Health approach breaks down the traditional silos of animal, environmental and human health and promotes collaboration across these disciplines to combat diseases which pose threats to humans and food supply systems. ACIAR recognises that this approach in low- and middle-income countries can have a profound impact on human health, livestock productivity and trade.

This study sought to adopt a One Health approach to developing a better understanding of the relationship between malnutrition and enteropathogen infections in infants and children in Dili, Timor-Leste. A key aim of the project was to strengthen capacity in human and animal public health within Timor-Leste and share the results with the community. The project also served as a pilot to establish the feasibility of conducting a larger study on the relationship between infant stunting and gastrointestinal infections in Timor-Leste.

We recruited a birth cohort of 60 infants from Hospital Nacional Guido Valadares (HNGV) over July-September 2019. The research team visited their homes when the infants were at 1, 3, 5, and 12 months of age, and collected demographic, socio-economic, and anthropometric data. A stool specimen was taken from the infant at each visit to test via multiplex PCR for 22 different bacterial, viral and parasitic enteropathogens, but specifically seeking detection and isolation of those bacterial enteropathogens that have been associated with intestinal damage and chronic inflammation in other studies: namely *Campylobacter, Salmonella* and *Shigella*. Animal and environmental specimens were also collected by teams from the Diagnostics Laboratory for Animal Health (DLAH) from some homes where infants were positive for *Campylobacter, Salmonella* or *Shigella* and culture tested for those specific enteropathogens at the DLAH.

To establish the enteropathogens associated with severe symptoms in children in Dili, we conducted enhanced laboratory surveillance through collecting faecal specimens from children aged 0-4 years who presented to Hospital Nacional Guido Valadares (HNGV) with severe acute malnutrition, diarrhoea with severe dehydration, or dysentery, and testing these specimens for enteropathogens.

The birth cohort showed considerable fluctuation in weight gain over the four visits, however 30% (8/27) of infants that were measured at all four visits had poor weight gain on at least one visit. Multiplex PCR testing is very sensitive and detects presence of pathogen nucleic acid but not necessarily viable pathogen. Enteric pathogens were detected by multiplex PCR in 74% (75/102) of stool specimens at the time of home visits, with the pathogen load increasing over time. Diarrhoeagenic *Escherichia coli* (DEC) strains were by far the most common pathogens detected, with 85 detections in 34 participants over the four visits. There were 15 *Campylobacter* infections detected in 14 participants, four *Salmonella* detections, two detections of STEC and two detections of *Shigella*/EIEC. No bacterial enteropathogens were successfully cultured from samples taken from the birth cohort. However the DLAH were able to successfully culture *Campylobacter* from animal and environmental specimens from the home environments of three birth cohort participants who had tested positive to *Campylobacte*r by PCR.

The median household size for the birth cohort household was 8 (range 3-15), with household sizes varying over the course of the study. Mothers predominantly were the

respondents to the questionnaires. There was an early uptake of supplementary feeding (41% at one month of age), and a decline in parents or guardians reporting that they boiled bottles between uses from visits 1 and 2 to visits 3 and 4, indicating a potential risk of contamination. All respondents reported some version of a pit latrine, 50% of respondents sourced some or all of their drinking water from ground sources such as bores, wells and springs, and a significant flood was experienced during the 2020 wet season. This combines to result in a strong risk for transmission of enteropathogen infections. Pathogen detection in both the birth cohort and participants in the hospital enhanced surveillance was also seasonal, with the highest number of pathogen detections in the wettest months of December to March. Household animals kept within the home/compound were common, with dogs, pigs, and chickens the most frequently reported animals.

The hospital enhanced surveillance included 159 children admitted to HNGV. Of the 148 participants for whom a stool specimen was collected, tested, and reported, 132 were diagnosed with severe acute malnutrition, 12 were diagnosed with diarrhoea with severe dehydration and four were diagnosed with dysentery. Multiplex-PCR testing showed that 90% (133/148) of participants were positive for at least one gastrointestinal pathogen and 86% (127/148) were positive for at least one of our target pathogens (*Campylobacter, Salmonella* and DEC). Pathogen detections in the hospital enhanced surveillance were broadly similar to the birth cohort, except for parasite infections, which were almost entirely absent in the birth cohort, and much more prevalent in the older participants (median 1.3 years) of the hospital enhanced surveillance.

Through this pilot study, we sought to build stronger links with public health in Timor-Leste and build local capacity in public health research, human and animal public health laboratory diagnosis, surveillance, and outbreak detection and investigation for enteropathogens in Timor-Leste. This occurred through recruiting and training a research team, providing steady throughput of samples at the laboratories and via structured training sessions provided by the research team at strategic points throughout the study. Capacity building in both research and laboratory skills was evidenced. The level of support provided by partners was extremely high. The Institute National da Saude provided considerable advice and support in designing the project. The Ministry of Agriculture and Fisheries (MAF) and the Diagnostics Laboratory for Animal Health (DLAH) were also eager to collaborate and the joint laboratory training session on *Campylobacter* isolation was very successful. The impact of detection of African Swine Fever in Timor-Leste in September 2019 consumed the time of DLAH officers, limiting capacity for animal and environmental sampling. Despite this, the collaboration with DLAH remains strong.

In conclusion, infants and children in Dili have a high enteric pathogen load and signs of growth retardation are exhibited from first months of life. Contributing factors are complex; however, our pilot study shows that both enteric pathogens, WASH, proximity of animals in the domestic setting, household crowding as well as an early move from exclusive breast feeding appear to be contributing factors. We have shown that it is feasible to undertake longitudinal field research in Timor-Leste and have developed strong relationships with partners in local health, veterinary, Ministry and non-governmental organisations.

Given the critical role that poultry plays in the lives of Timor-Leste families, we recommend a larger-scale study to explore infant and child dietary practices, food safety and environmental hygiene conditions in relation to community poultry production with a focus on the risk of *Campylobacter* and *Salmonella.* This project should consider targeted interventions in urban and rural settings in Timor-Leste, utilising a One Health collaborative approach.

3 Acronyms

ANU – Australian National University

BioFire – The BioFire® FilmArray® Gastrointestinal Panel

DEC – diarrhoeagenic *Escherichia coli*

- EAEC – enteroaggregative *Escherichia coli*
- EPEC – enteropathogenic *Escherichia coli*
- ETEC – enterotoxigenic *Escherichia coli*
- *Shigella*/EIEC – Shigella/enteroinvasive *Escherichia coli*
- STEC – Shiga toxin-producing *Escherichia coli*
- *E. coli* O157 – specific O157 serogroup of Shiga-toxin producing *Escherichia coli*

DLAH – Diagnostics Laboratory for Animal Health / Laboratóiu Diagóstiku Saúde Animál

DWSD – diarrhoea with severe dehydration

GCP – Good Clinical Practice

HNGV – Hospital Nacional Guido Valadares

IQR – interquartile range

MAF – Ministry of Agriculture and Fisheries/Ministeriu Agrikultura no Peskas

MoH – Ministry of Health/Ministério da Saúde

INS – Institute Nacional da Saúde/National Institute of Health

IN-HRETC - Institute National of Health-Research Ethics & Technical Committee

Menzies – Menzies School of Health Research

NHL – National Health Laboratory

PCR – Polymerase Chain Reaction

REDCap – Research Electronic Data Capture

SAM – severe acute malnutrition

Timor-Leste – República Democrática Timor-Leste

WASH – water, sanitation, and hygiene practices

WAZ – weight-for-age Z score

WHZ – weight-for-length/height Z score

WHO – World Health Organization

WHO CDC – Communicable Disease Control/Departementu Controlo de Doencas Contagiosas

STRONG-TL – Surveillance, Training, Research Opportunities, National Guidelines for communicable disease control in Timor-Leste

PULSA study – Pneumonia and Malnutrition study at HNGV

4 Introduction

Stunting (low height/length for age), wasting (low weight for height/length) and underweight (low weight for age) are internationally recognised markers of child malnutrition and are commonly observed in children in low- and middle-income countries. There are many socio-economic and environmental factors that contribute to malnutrition; however, one that has only been explored recently is enteropathogen infection. There is growing evidence of an association between infectious gastroenteritis and malnutrition in low-income countries (1-5). Enteric bacterial infections contracted in infancy can damage the lining of the small and large intestines, reducing the absorption of nutrients from food and promoting chronic inflammation, leading to malnutrition and compromising the immune system (1, 2). These bacterial enteropathogens include enteropathogenic variants of normal microflora such as entero-toxigenic *Escherichia coli* (ETEC) and *Shigella* species (3). Recently, *Campylobacter* has been shown to be associated with stunting among children aged 0-6 years in Peru and in a large birth cohort study in eight sites in South America, sub-Saharan Africa and South Asia (4, 5). The MAL-ED study found 84.9% of children studied (n=1606) had at least one *Campylobacter*-positive stool sample by age one, and after adjusting for confounders, that children diagnosed with multiple *Campylobacter* infections during the study were significantly more likely to be stunted compared to children with one or no *Campylobacter* infections (4).

Campylobacter is the most common cause of bacterial gastroenteritis globally, causing an estimated 166 million episodes of diarrhoea and >38,000 associated deaths annually (6). Control of *Campylobacter* requires a true One Health approach. *Campylobacter* is an environmental contaminant of soil and water; a zoonosis of wild birds, companion animals, and farmed animals such as pigs and poultry; and a foodborne infection transmitted through cross-contamination of raw ingredients and poor cooking practices.

Since independence in 2002, the República Democrática Timor-Leste (Timor-Leste) has moved from a post-conflict country to a lower-middle-income country (7). A report by UNICEF in cooperation with the General Directorate of Statistics of the Ministry of Finance found that in 2013, the prevalence of malnutrition in children under five years of age has significantly decreased since 2009-10, but remained amongst the highest in the world: stunting (51.9%); wasting (10.8%); and underweight (38.1%) (7). Other more recent publications have confirmed that malnutrition remains a significant issue in Timor-Leste (8-10).

Diarrhoea is a major public health concern in Timor-Leste and is the second most common disease identified in children less than five years of age, rising from 75,000 reported cases in 2014 to more than 110,000 in 2016 (11). Despite this, the aetiology of diarrhoeal disease is poorly characterised, due to a lack of diagnostic capacity within the country (11). There are few studies of the incidence rates of enteropathogen infections in Oceania, but a recent study in Fiji detected *Campylobacter* (using PCR) from 59.2% (n=241/408) of diarrhoeal faecal samples from two major hospital pathology laboratories. Samples from children aged less than five accounted for 21.6% of these 241 positive cases (12). Acute diarrhoea and bloody diarrhoea, both symptoms of gastrointestinal infection, are listed as numbers two and ten respectively, in the top ten diseases routinely reported in Timor-Leste (8). There have been no published investigations into the association between enteropathogen infections and stunting in the Oceania region. Therefore, there is a gap in current knowledge on the prevalence of enteropathogen infections in Timor-Leste and their potential association with malnutrition in young children.

5 Objectives and Activities

The aim of this study was to address the following research questions on enteropathogens and stunting in Timor-Leste:

1. What is the current burden of gastrointestinal infections due to enteropathogens in infants in a specific community in Timor-Leste?
2. What social, environmental, food security and food safety issues are associated with enteropathogen infections in this community?
3. What enteropathogen infections are present in children who present to Hospital Nacional Guido Valadares with diarrhoea or are admitted with acute malnutrition?
4. What is the population prevalence of stunting in infants of this community and how does it compare to national estimates?

These research questions were addressed through the following objectives and activities.

5.1 Objectives:

1. Developing a better understanding of the relationship(s) between malnutrition and enteropathogen infections in infants and children in Timor-Leste.
2. Determining the potential contribution of social, zoonotic, and environmental factors in human enteropathogen infections in a small sample of infants in Timor-Leste.
3. Building capacity in human and animal public health laboratory diagnosis, surveillance, and outbreak detection and investigation for enteropathogens in Timor-Leste.

5.2 Activities:

1. Conduct a pilot study in a birth cohort recruited from maternity records of the Hospital Nacional Guido Valadares in Dili Timor-Leste, including qualitative and anthropometric measures and faecal testing for enteropathogens;
2. Conduct enteropathogen testing of companion animals and livestock of study families, and of the water and environment close to the homes of children in the birth cohort;
3. Conduct testing of faecal specimens from children presenting with symptoms of gastroenteritis and children admitted with severe acute malnutrition from the Hospital Nacional Guido Valadares;
4. Develop strong partnerships with relevant governmental and non-governmental stakeholders in Timor-Leste;
5. Build local capacity in public health microbiology via increased throughput at the National Health Laboratory and the Diagnostics Laboratory for Animal Health; and
6. Build local capacity in public health surveillance, and outbreak detection and investigation via delivery of targeted training seminars and assisted investigation of family clusters of gastroenteritis detected during the birth cohort study.

6 Methodology

The project incorporated four main elements:

1. A pilot birth cohort study examining indicators of stunting and the association with enteropathogen carriage from 60 families recruited sequentially from births recorded at Hospital Nacional Guido Valadares and living within a 10km radius of the hospital;
2. Testing of faecal specimens for pathogens from children presenting with diarrhoea or admitted with severe acute malnutrition to the Hospital Nacional Guido Valadares;
3. Testing of animal, water and environmental specimens collected from participants' homes and local area; and
4. Capacity building training of Timor-Leste public health workforce around: laboratory detection of enteropathogens; diagnosis and treatment of enteropathogens; and investigation of diarrhoeal disease outbreaks.

To facilitate linkages with Timor-Leste institutions, project leaders met with local staff on initial scoping site visits in December 2018 and early 2019 to plan the pilot study. The research team then recruited and trained staff in Timor-Leste to manage the project and liaised with them on a weekly basis via mobile phone. In addition, three further field visits were undertaken by the ANU team to work with the local team to monitor study progress, clean data, address implementation issues and meet with local partner organisations. The local research team were supported on a day-to-day basis by the STRONG-TL investigators and Menzies School of Health Research resident staff in Dili.

6.1 Study locations

Hospital Nacional Guido Valadares (HNGV) is both the largest hospital and the only tertiary referral hospital in the country and is located in Dili, the capital city of Timor-Leste. HNGV has a catchment population of approximately 500,000 and approximately 4,500 births per year in the maternity unit (13-15). The National Health Laboratory (NHL) is located next to HNGV, convenient for transport and testing of specimens. The Diagnostics Laboratory for Animal Health (DLAH) is a short drive from HNGV.

6.2 Birth cohort

Supported by maternity unit staff, the local research team recruited 60 newborn infants from maternity wards at HNGV. Sampling was opportunistic, with all women who had a live birth in HNGV from 11 July 2019 offered the chance to enrol their infant in the study. Recruitment ceased once 60 participants were recruited, which occurred on 3 September 2019. Mothers and newborn babies were routinely discharged on day one or day two following delivery. The research team recruited and enrolled newborns following a standard informed consent process, following training in Good Clinical Practice (GCP) for research. Demographic details were collected at this time, and home address confirmed by the research team in the week following discharge.

6.2.1 Birth cohort inclusion and exclusion criteria

Newborn infants, 38 weeks or more gestation born at HNGV were eligible for inclusion. Families were asked if their home address was within a 10 km radius of HNGV, to both reduce travel for the research team undertaking the home visits and to lessen the attrition from the study due to difficulty in reaching families during times of heavy rain and floods.

6.2.2 Home visits

Home visits were undertaken when the infant was approximately 1, 3, 5, and 12 months of age. The research team rode personal motorbikes to attend home visits and were reimbursed for fuel. The research team were issued with a calibrated portable baby scale and a standardised mat for measuring height, stool sample pots, an insulated ice-chest for collecting and transporting stool specimens, and a sturdy and secure backpack for transporting it all. The researchers recorded study data on an android tablet and synchronised online with a linked laptop upon return to the study office.

At each home visit the research team completed an extensive questionnaire, based on the validated Timor-Leste Demographic and Health Survey 2016, with the parents and/or carers of the enrolled infant. The questionnaire determined feeding practices, household size, water, sanitation, and hygiene (WASH) practices and accessibility, and household animal ownership and animal containment. In addition, all infants were weighed and measured using a standardised procedure (in which the research team was trained), using calibrated portable scales and a standardised length infant measurement mat.

A stool sample was collected from the infant either at the time of the field visit, or in the following days in a sterile stool specimen container that the research team then transported to the laboratory for PCR and culture testing (see 5.4 below). Parents were provided with oral and printed visual instructions in Tetum to ensure that stool samples were appropriately collected and stored prior to transport. Most enteropathogen infections require supportive treatment only, so no treatment was offered by the research team.

We anticipated a 33% attrition rate in the birth cohort based on other similar studies to give us an effective sample size of 40 children for the pilot study. To improve retention in the study, we provided a small incentive of mobile phone recharges (USD$2.00) for participation at each household visit. The research team communicated with the families enrolled in the study by mobile phone throughout the entire study period. Any laboratory testing disposables that were underutilised by the birth cohort were repurposed to support the hospital enhanced laboratory surveillance.

6.2.3 Animal and Environmental Sampling

When specific environmental/zoonotic enteropathogens (*Campylobacter*, *Salmonella*, or Shiga toxin-producing *Escherichia coli*) were detected during testing of birth cohort specimens, the research team sought permission from the family to return and collect specimens from animals and the environment around the families' home. If permission was granted, the research team joined with researchers from the Diagnostics Laboratory for Animal Health (DLAH), to revisit the home. DLAH researchers took swabs of fresh animal faeces and from animals using standard procedures. These samples were then tested by culture for *Campylobacter*, *Salmonella*, and *Shigella* species at DLAH.

6.3 Hospital based enhanced laboratory surveillance

There have been several studies showing that diarrhoeal disease is an important issue in Timor-Leste but few studies have included pathogen testing to show which enteropathogens are causing disease (11). To further develop linkages with the Hospital Nacional Guido Valadares (HNGV) and gain a better understanding of the enteropathogens causing illness in the Dili community and asymptomatic carriage in malnourished children, we planned to conduct diagnostic testing of 150 faecal specimens from children aged 0-9 years admitted to HNGV with symptoms of severe acute malnutrition, diarrhoea with severe dehydration, or dysentery. However after discussions with the HNGV paediatrics team it became evident that we should focus on the children most impacted by stunting and most often admitted with severe disease—infants and children aged 0-4 years—and still easily meet our recruitment target. Rapid diagnosis of stool specimens by PCR in this cohort would augment clinical diagnosis and treatment

regimes, and potentially benefit clinical practice at HNGV. Thus the study protocol was changed to focus on this age group.

6.3.1 Hospital participant inclusion and exclusion criteria

The criteria for recruitment into the hospital enhanced surveillance were based on WHO criteria for the diagnosis of severe acute malnutrition (SAM), severe diarrhoea and/ or severe dysentery, which was adapted to align with standard HNGV paediatrics team protocols (Table 1). Children aged five years or older, or who resided outside of Dili, or who were not citizens of Timor-Leste were excluded.

Table 1: Inclusion criteria for recruitment of hospitalised cases aged 0-4 years.

Diagnosis	Clinical Criteria	Project Diagnosis Code
Diarrhoea Admissions at HNGH	Severe acute diarrhoea that requires fluid resuscitation	Diarrhoea with severe dehydration
	Severe dysentery that requires treatment with Ceftriaxone	Dysentery
Malnutrition	Below -3 Z score of the median WHO growth standards, by visible severe wasting, or presence of nutritional oedema*. Prioritise admitted infants and children < 2years	Severe acute malnutrition (SAM)

*https://apps.who.int/nutrition/topics/severe_malnutrition/en/index.html

Research team members attended morning medical rounds with the HNGV paediatric team to identify children meeting the inclusion criteria and then liaised with the clinicians to obtain a stool specimen and record vital statistics. Stool samples from identified eligible infants and children were collected by the ward nurses. The paediatrician notified the research team, who organised transport of the specimens to the laboratory.

6.4 Laboratory testing - both study arms

6.4.1 National Health Laboratory

A rate limiting factor on the development of environmental and public health laboratory capacity in Timor-Leste was the lack of a steady supply of specimens to test. This Project provided a steady but manageable flow of specimens by staggering sample collection to ensure consistent throughput of faecal samples through the National Health Laboratory (NHL). NHL staff were paid USD$2.00 per sample to conduct testing for this study. They tested all stools from the birth cohort and hospital enhanced surveillance using a BioFire FilmArray Gastrointestinal Panel polymerase chain reaction (PCR) assay capable of detecting 22 pathogens and parasites, including: *Campylobacter* spp., *Clostridium difficile* (toxin A/B), *Plesiomonas shigelloides*, *Salmonella* spp., *Vibrio* spp., *Vibrio cholerae*, *Yersinia enterocolitica*, enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC) (including specific detection of *E. coli* O157), *Shigella* spp./enteroinvasive *E. coli* (*Shigella*/EIEC),

Cryptosporidium spp., *Cyclospora cayetanensis*, *Entamoeba histolytica*, *Giardia lamblia*, adenovirus F 40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus (16).

Specimens positive by PCR for selected enteric bacteria that have been shown to damage the intestines and promote chronic inflammation, including *Campylobacter, Salmonella* and *Shigella*/EIEC, were reflex cultured on appropriate selective media.

6.4.2 Diagnostics Laboratory for Animal Health

Environmental and animal specimens collected by the DLAH teams were then tested by culture for *Campylobacter*, *Salmonella*, and *Shigella* on appropriate selective media at DLAH. The BioFire assay is not validated for use on animal or environmental specimens.

6.5 Analysis of data

The ANU team trained the local research manager and nurse in Good Clinical Practice (GCP) for research in human health, using modified training materials from the Global Health Research Network.[1] The research team collected and entered all data into a password protected Research Electronic Data Capture (REDCap) online database (17), stored on an ANU secure server. Data cleaning was completed both during ANU team member visits and online in liaison with the Timor-Leste research manager. Data were exported into STATA 15.1(18) for further data cleaning and analysis. A descriptive analysis was undertaken using R Studio statistical software (19) for both the birth cohort and hospital arm of the study. To assess for malnutrition, anthropometric measures were converted to weight-for-length/height Z (WHZ) scores for the birth cohort and weight-for-age Z (WAZ) scores for the hospital enhanced surveillance. These were also mapped against the WHO child growth standards[2] using the R package “anthro”. Categorical variables were compared using Fisher’s exact tests, χ^2 test for trend, and Odds ratios where appropriate, and p <0.05 was considered significant for all tests.

6.6 Ethics approval

The Institute National of Health-Research Ethics & Technical Committee ref: 462/MS-INS/GDE/IV/2019 on 03/04/2019 and the Australian National University ref: 2019/221 on 08/05/2019, granted ethics approval for this research study.

[1] *https://globalhealthtrainingcentre.tghn.org/elearning/*

[2] *https://www.who.int/tools/child-growth-standards/standards*

7 Key results and discussion

7.1 Objective 1: Developing a better understanding of the relationship(s) between malnutrition and enteropathogen infections in infants and children in Timor-Leste.

To achieve this objective, we employed a two-pronged strategy: recruiting a birth cohort and participants for hospital enhanced laboratory surveillance.

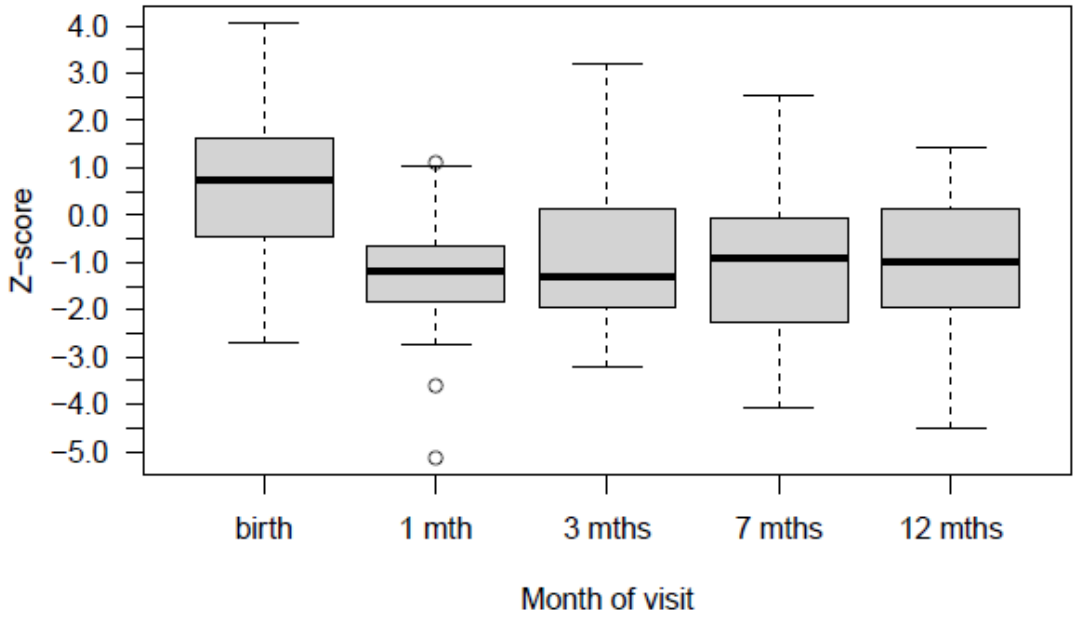
7.1.1 Birth Cohort

Thirty-two males and 28 females were recruited. There was a similarly high rate of study attrition as we expected, with a 52% retention rate to the final (fourth) visit (32/60 participants).

Height and weight

The 60 newborns recruited presented at birth with a median weight-for-height Z-score (WHZ) of 0.90 (IQR -0.32 to 1.78). However the median WHZ dropped to -1.2 (IQR -1.8 to -0.6) at the first home visit and remained low throughout the infants' first year of life. By the final home visit at approximately 12 months of age, the median WHZ was -0.94 (IQR -1.76 to 0.16) (Figure 1).

Figure 1: Box plot of birth cohort median weight for length/height Z-scores at each home visit.



There was considerable fluctuation in individual WHZ over the course of the study, but no participant's WHZ consistently decreased across the four home visits. However, of the 27 infants who were weighed and measured at all four home visits, 30% (8/27) exhibited poor weight gain (WHZ of -3) on at least one visit.

Figure 2 shows the birth cohort data mapped against the length-for-age standards for the first 450 days and Figure 3 shows the birth cohort data mapped against the weight-for-age standards for the first 450 days.

Figure 2: Birth cohort participants measured length at each home visit and WHO length-for-age standard for first 450 days of life

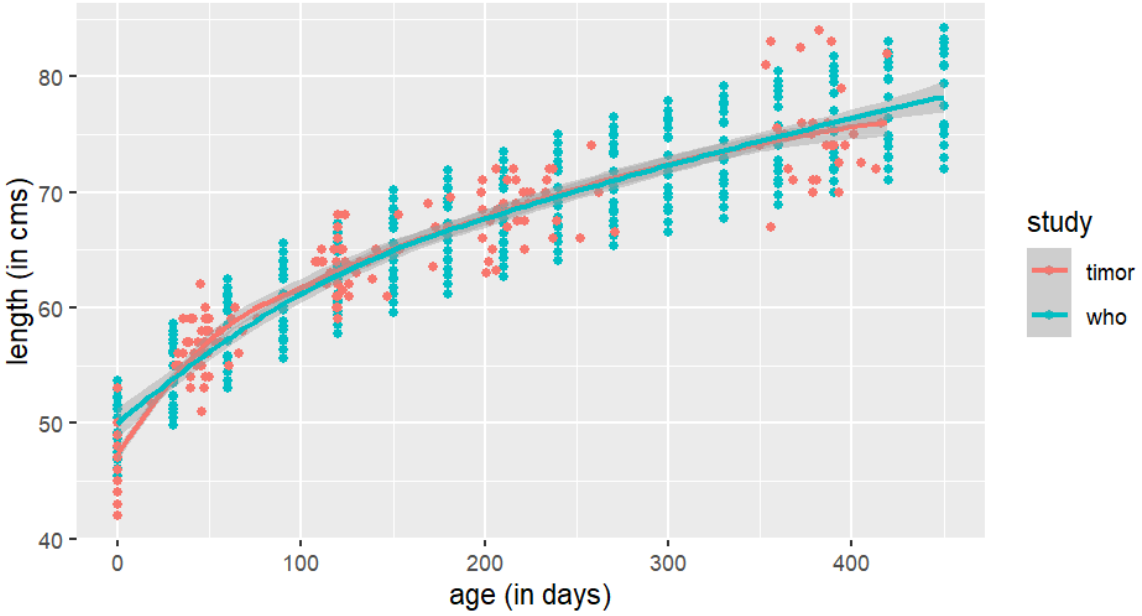
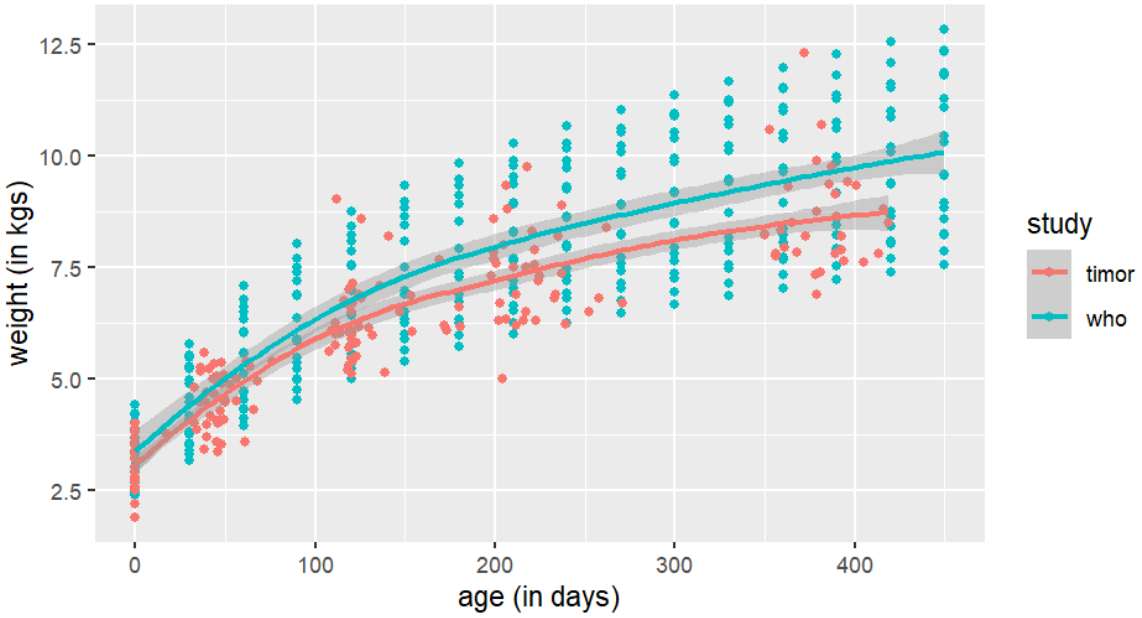


Figure 3: Birth cohort participants measured weight at each home visit and WHO weight-for-age standard for first 450 days of life



Figures 2 and 3 demonstrate that in comparison to the WHO standards, the birth cohort participants are approximately in the 50th percentile for length for age, but approximately in the 25th percentile for weight for age.

Stool specimen testing

Across the entirety of the birth cohort home visits, a total of 141 stool specimens were collected, all of which were tested. This was a great result for parent/guardian collected specimens.

Overall, enteric pathogens were detected by multiplex PCR in 69% (97/141) of stool specimens at the time of home visits. The pathogen load detected in the birth cohort (healthy infants visited in the home) increased over time. This increase was statistically significant for *Campylobacter, Salmonella*, Diarrhoeagenic *Escherichia coli* (DEC) strains, parasites and viruses (Table 2). Each of the 22 children that submitted a stool specimen for testing at each home visit was PCR positive for at least one enteropathogen, and 41% (9/22) of these children were PCR positive for at least one enteropathogen at all four home visits.

Table 2: Number of pathogens detected* by multiplex PCR and percentage positive in the birth cohort at each home visit.

Pathogen	Visit 1 (n=45)	Visit 2 (n=34)	Visit 3 (n=36)	Visit 4 (n=26)	*p* value†
Any pathogen	16 (35.6)§	25 (73.5)	33 (91.7)	23 (88.5)	<0.01
Campylobacter spp.	3 (6.7)	1 (2.9)	3 (8.3)	8 (30.8)	<0.01
DEC	12 (26.7)	22 (64.7)	31 (86.1)	20 (76.9)	<0.01
Viruses**	0 (0.0)	6 (17.6)	14 (38.9)	9 (34.6)	<0.01
Parasites††	0 (0.0)	0 (0.0)	1 (2.8)	3 (11.5)	<0.01
Clostridium difficile	1 (2.2)	4 (11.8	4 (11.1)	4 (15.4)	0.06
Salmonella spp.	0 (0.0)	0 (0.0)	4 (11.1)	0 (0.0)	0.20
Plesiomonas shigelloides	0 (0.0)	0 (0.0)	1 (2.8)	0 (0.0)	0.53
Vibrio spp.	1 (2.2)	0 (0.0)	3 (8.3)	0 (0.0)	0.72
Yersinia enterocolitica	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	n/a

*Multiple pathogens can be detected per specimen; †χ^2 test for trend; §results reported as number positive (percent positive); DEC – Diarrhoeagenic *Escherichia coli* **adenovirus F 40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus; ††*Cryptosporidium* spp.*, Cyclospora cayetanensis, Giardia lamblia*.

In this cohort, most infants that submitted a stool specimen that was positive for an enteropathogen were either asymptomatic (solid stool) or exhibiting minimal symptoms (semi-solid stool) (Table 3), meaning presentation to health services and subsequent clinical diagnosis of gastroenteritis would be very unlikely. One stool specimen was indicative of bloody diarrhoea (dysentery). This specimen was PCR positive for EAEC and EPEC.

Table 3: Number of pathogens detected* by multiplex PCR, percentage positive, and stool consistency in the birth cohort over the entire study period (n=141).

Pathogen	Solid	Semi-solid	Watery	Watery & bloody	Total
Campylobacter spp.	4 (2.8)†	8 (5.7)	3 (2.1)	0 (0.0)	15 (10.6)
Salmonella spp.	1 (0.7)	3 (2.1)	0 (0.0)	0 (0.0)	4 (2.8)
DEC	8 (5.7)	61 (43.3)	15 (10.6)	1 (0.7)	85 (60.3)
Vibrio *spp*.	1 (0.7)	3 (2.1)	0 (0.0)	0 (0.0)	4 (2.8)
Clostridium difficile	1 (0.7)	11 (7.8)	1 (0.7)	0 (0.0)	13 (9.2)
Plesiomonas shigelloides	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)	1 (0.7)
Yersinia enterocolitica	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Parasites§	2 (1.4)	1 (0.7)	1 (0.7)	0 (0.0)	4 (2.8)
Viruses**	2 (1.4)	21 (14.9)	4 (2.8)	0 (0.0)	29 (20.6)

*Multiple pathogens can be detected per specimen; †results reported as number positive (percent positive); DEC – Diarrhoeagenic *Escherichia coli;* §*Cryptosporidium* spp.*, Cyclospora cayetanensis, Giardia lamblia*; **adenovirus F 40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus

Campylobacter was a key focus of our study, as it is the enteric pathogen most associated with stunting in other studies. Over the 12 months of the study, there were 15 *Campylobacter* infections detected in 14 participants. Eight *Campylobacter* infections were detected in the visit at 12 months, compared to seven for the previous three visits combined (see Table 2). One participant was diagnosed with *Campylobacter* twice: at 1 month and at 12 months of age. Co-infection was also common: 14/15 stools positive for *Campylobacter* were also positive for at least one other pathogen. However, only three of the 15 detections were in watery stool, indicating the majority were exhibiting minor symptoms or asymptomatic. Previous studies have shown similar incidence in early infections in asymptomatic infants (20).

Salmonella was detected in four infants at the third home visit. Each of these four specimens were also PCR positive for at least two other pathogens, although all four were detected in solid (1) or semi-solid (3) stools. There were also 13 detections of *Clostridium difficile* with specimens from three participants being positive for *C. difficile* at two home visits. One specimen was positive for *Plesiomonas shigelloides*.

Diarrhoeagenic *Escherichia coli* (DEC) strains were by far the most common pathogens detected, with 85 detections in 34 participants over the four visits. Most participants (31/34) with a DEC detection had multiple detections, and six participants submitted a stool positive for a DEC strain at all four visits, indicating either multiple infections or chronic carriage. Of these six participants, none presented with the same pattern of DEC strains at each visit, likely indicating multiple infections; however, EAEC was detected in 23/24 stool specimens for these participants, potentially indicating chronic carriage. This is consistent with other studies that have found DEC strains to be common causes of both acute and persistent paediatric diarrhoea in low- and middle-income countries (21, 22). There were two detections of Shiga-toxigenic *Escherichia coli* (STEC) in visit 4, and two detections of *Shigella*/EIEC, one each in visit 1 and visit 3. All of these were in different participants. These pathogens can cause severe infections, particularly in infants, but none of the four specimens were indicative of diarrhoea.

Seasonality

The progression of the home visits and the pathogens detected are displayed in Figure 4. This Figure demonstrates the low levels of pathogen detected per specimen for the first home visit, and the substantial increase in subsequent visits, peaking at the 3rd home visit in February with a ratio of 1.6 pathogens detected per specimen tested.

Figure 4: Detections of gastrointestinal pathogens by multiplex PCR in the birth cohort home visit specimens vs specimens tested, July 2019 to September 2020

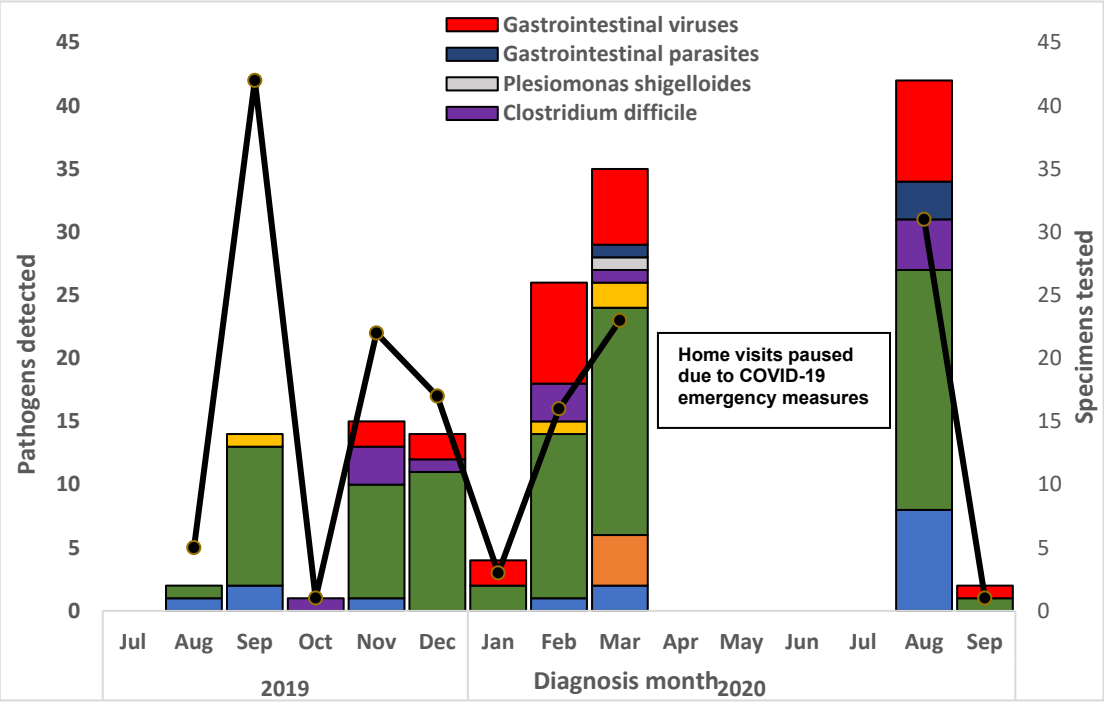


Table 4: Number of pathogens detected* by multiplex PCR and percentage positive in the birth cohort by season

Pathogen	Wet Season (n=52)	Dry Season (n=89)	Odds Ratio (95% CI)	*p* value†
DEC	44 (84.6)§	41 (46.1)	6.4 (2.6-17.5)	<0.01
Viruses**	18 (34.6)	11 (12.4)	3.7 (1.5-9.7)	<0.01
Salmonella spp.	4 (7.7)	0 (0.0)	n/a	0.02
Vibrio *spp.*	3 (5.8)	1(1.1)	5.3 (0.4-285.5)	0.14
Campylobacter spp.	3 (5.8)	12 (13.5)	0.4 (0.1-1.6)	0.26
Plesiomonas shigelloides	1 (1.9)	0 (0.0)	n/a	0.37
Clostridium difficile	5 (9.6)	8 (9.0)	1.1 (0.3-4.0)	1.00
Parasites††	1 (1.9)	3 (3.4)	0.6 (0.0-7.2)	1.00
Yersinia enterocolitica	0 (0.0)	0 (0.0)	n/a	n/a

*Multiple pathogens can be detected per specimen; †Fisher's exact 2 tailed; DEC – Diarrhoeagenic *Escherichia coli;* §results reported as number positive (percent positive); **adenovirus F 40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus; ††*Cryptosporidium* spp., *Cyclospora cayetanensis, Giardia lamblia*

Figure 4 and Table 4 demonstrate that there was seasonality in pathogen detections in the birth cohort. However it is important to note that there was no testing in the dry season months of May to July, or the wet season month of April, and very little testing in the dry season month of October. There was a statistically significant positive association found between the wet season and detections for: enteric viruses, odds ratio (OR) 3.7, 95% confidence interval (95%CI) (1.5-9.7), p<0.01; and DEC, OR 6.4, 95%CI (2.6-17.5), *p*<0.01. All four *Salmonella* detections were in the wet season month of March, but an odds ratio could not be calculated due to a zero cell. Detections for *Campylobacter* were higher in the dry season although this was not statistically significant. Considering enteric viruses and DEC pathotypes individually, a statistically significant positive association was found between the wet season and detections for EAEC, ETEC, EPEC, norovirus and sapovirus (see Supplementary Table S2).

Isolation of bacterial enteropathogens

No bacterial enteropathogens were cultured from samples taken from the birth cohort. This is reflective of a number of factors. Firstly, there were very few detections of *Salmonella* and *Shigella*/EIEC by PCR (four and two detections respectively) limiting chances for culture. Secondly, there were difficulties in isolating *Campylobacter* without a 42^0C incubator. Finally, as only 17% of stools tested were indicative of diarrhoea, most infections were likely present at low concentrations. That is, the much higher sensitivity of PCR testing was likely detecting dead pathogens or live pathogens at levels too low to be cultured. Neither of the two specimens where *Shigella*/EIEC was detected were indicative of diarrhoea, so it is likely they were EIEC which produces milder symptoms and is not able to be cultured on *Shigella* selective media.

Animal and environmental testing

For each participant where *Campylobacter, Salmonella,* or diarrhoeagenic *E. coli* were detected, the research team contacted the family and offered to return with a team from the Diagnostics Laboratory for Animal Health (DLAH) to take animal and environmental specimens to test for these enteropathogens.

Three visits were conducted during the course of the first home visit with the findings that:

1. Participant was PCR positive for *Campylobacter*, and the family reported ownership of one chicken. The DLAH team sampled fresh faeces from the chicken and were able to isolate *Campylobacter*.
2. Participant was PCR positive for *Campylobacter*, and the family reported ownership of five dogs, three pigs, and two chickens. The DLAH team sampled fresh faeces from chicken and pigs and were able to isolate *Campylobacter*.
3. Participant was PCR positive for *Campylobacter*, EAEC, EPEC and ETEC, and the family reported ownership of three pigs and eight chickens. The DLAH team sampled fresh faeces from chicken and pigs and were able to isolate *Campylobacter*.

The success of the DLAH team in isolating *Campylobacter* (the first time they had done so) is commendable and demonstrates the feasibility of future collaborations between public health and animal health in Timor-Leste to investigate One Health issues.

7.1.2 Hospital enhanced laboratory surveillance

The research team enrolled 159 children aged 0-4 years, and one child aged seven years, who were admitted to HNGV with a diagnosis of severe acute malnutrition, diarrhoea with severe dehydration or dysentery. Of these, two participants were discharged before a sample could be collected, and a result was not recorded for nine participants due to insufficient stool specimen submitted, failed verification checks, or other process issues.

Height and weight

All 148 participants were demonstrably stunted exhibiting a median weight-for-age z-score (WAZ) of -3.8 (IQR -5.1– -2.9) (Table 5).

Table 5: Breakdown of Hospital enhanced surveillance by age, sex and Z-score

Sex	n	Mean age in years (range)	Median WAZ (IQR)
male	68	1.6 (0.2 to 4.6)	-4.2 (-6.0 to -3.1)
female	79	1.4 (0.1 to 7.5)†	-3.6 (-4.8 to -2.9)
Total	147*	1.5 (0.1 to 7.5)†	-3.8 (-5.1 to -2.9)

*Sex wasn't recorded for one participant; †One participant aged 7 years was recruited

Diagnosis

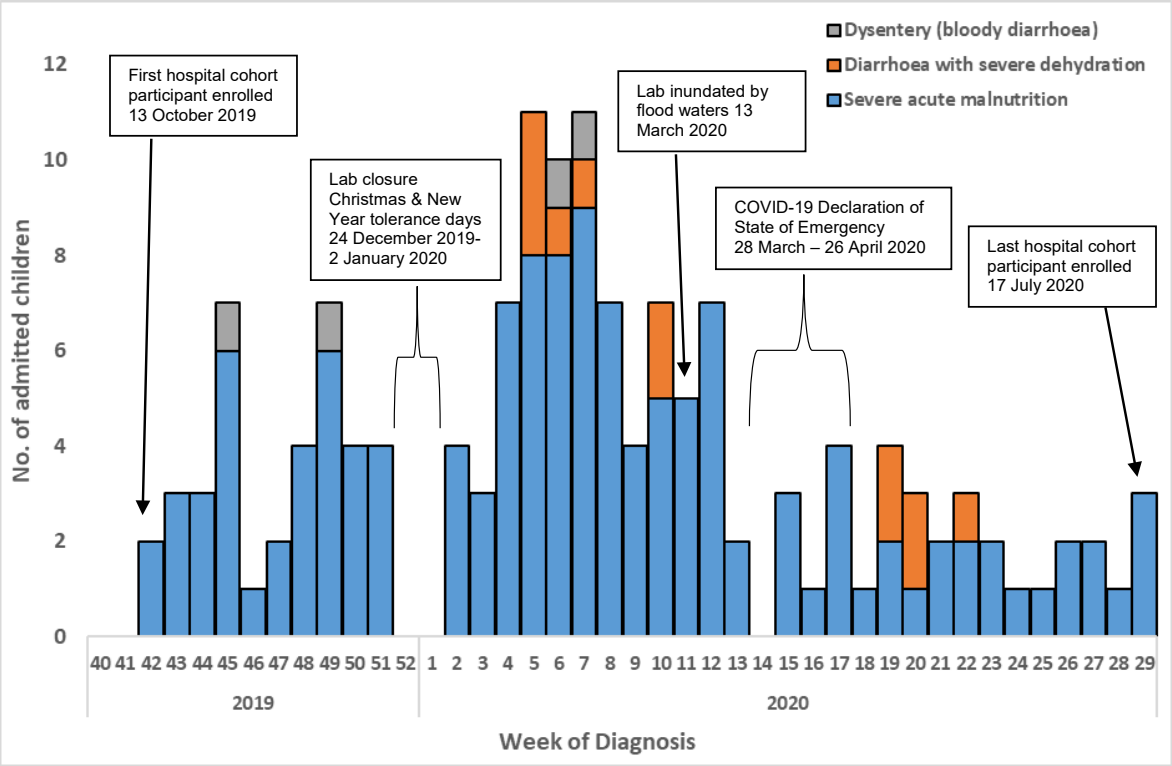
Of the 148 participants for which a stool specimen was collected, tested, and reported, the majority (89%, 132/148) were admitted with a diagnosis of severe acute malnutrition (SAM). Twelve participants were admitted with a diagnosis of diarrhoea with severe dehydration and four participants were diagnosed with dysentery (Table 6).

Table 6: Number of gastrointestinal pathogens detected by multiplex PCR and percentage positive, by admission diagnosis in the hospital enhanced surveillance (n=148)

Admission diagnosis	No pathogens Detected (%)	One pathogen detected (%)	Multiple pathogens detected (%)
Severe Acute Malnutrition	14 (9.5)	42 (28.4)	76 (51.4)
Diarrhoea with severe dehydration	1 (0.7)	2 (1.4)	9 (6.1)
Dysentery	0 (0.0)	2 (1.4)	2 (1.4)
Totals	15 (10.1)	46 (31.1)	87 (58.8)

Figure 5 shows the spread of participant recruitment in the hospital enhanced surveillance, in the 10-month 2019-20 period. There was an average of 3.2 diagnoses of severe acute malnutrition per week, peaking at nine diagnoses in week 7 of 2020. Diarrhoea with severe malnutrition was much less common, with 12 diagnoses but exhibited some clustering, with five diagnoses across weeks 5-7 coinciding with the three highest weeks for severe acute malnutrition and two of the four dysentery diagnoses.

Figure 5: Hospital enhanced laboratory surveillance participants aged 0-4 years* admitted to HNGV by diagnosis 1 October 2019-18 July 2020



*1 participant recruited was 7 years of age

Stool specimen testing

Table 7: Number of pathogens detected* by multiplex PCR and percentage positive in the hospital enhanced laboratory surveillance, by diagnosis

Pathogen	DSWD or dysentery (n=16)	SAM (n=132)	Odds Ratio (95% CI)	*p* value†
Campylobacter spp.	3 (18.8) §	34 (25.6)	0.7 (0.1-2.6)	0.76
Salmonella spp.	1 (6.3)	4 (3.0)	3.5 (0.0-23.4)	0.44
DEC	15 (93.8)	107 (80.5)	3.5 (0.5-153.3)	0.31
Vibrio spp.	2 (12.5)	3 (2.3)	6.14 (0.5-57.3)	0.09
Clostridium difficile	0 (0.0)	6 (4.6)	0.0 (0.0-5.4)	1.00
Plesiomonas shigelloides	0 (0.0)	1 (0.8)	n/a	1.00
Yersinia enterocolitica	0 (0.0)	0 (0.0)	n/a	n/a
Parasites**	3 (18.8)	47 (35.3)	0.4 (0.1-1.6)	0.26
Viruses††	5 (31.3)	34 (25.6)	1.3 (0.3-4.5)	0.76

*Multiple pathogens can be detected per specimen; DWSD – diarrhoea with severe dehydration, SAM –severe acute malnutrition; †Fisher's exact 2 tailed; §results reported as number positive (percent positive); DEC – Diarrhoeagenic *Escherichia coli;* ***Cryptosporidium* spp.*, Cyclospora cayetanensis, Giardia lamblia;* ††adenovirus F 40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus.

Testing showed that 90% (133/148) of participants were multiplex-PCR positive for at least one gastrointestinal pathogen and 86% (127/148) were positive for at least one bacteria known to promote intestinal inflammation and malabsorption of nutrients *(Campylobacter, Salmonella* and diarrhoeagenic *Escherichia coli*). A range of additional enteric bacteria, parasites and viruses were also detected in stool samples obtained from these children (Table 7). There was no statistically significant relationship between pathogen detection and diagnosis except for the individual DEC pathotype *Shigella*/EIEC. The odds of detection of Shigella/EIEC by PCR were 3.7 times higher in those who were diagnosed with DSWD or Dysentery compared to those diagnosed with SAM (OR 3.7, 95%CI 1.0-12.3, $p<0.05$) (Appendix Table S3).

Table 8: Comparison of stool consistency and admission diagnosis in the hospital enhanced surveillance (n=148)

Admission diagnosis	Solid	Semi-solid	Watery	Watery & bloody	Total
Severe Acute Malnutrition	14	108	10	0	132
Diarrhoea with severe dehydration	2	9	1	0	12
Dysentery	0	4	0	0	4
Total	16	121	11	0	148

Table 8 demonstrates the complex clinical picture presented by the participants of the enhanced laboratory surveillance. Of those participants diagnosed with severe acute malnutrition 7.6% (10/132) submitted a watery stool sample indicative of gastroenteritis. Conversely, only one of the participants diagnosed with diarrhoea with severe dehydration submitted a watery stool, and none diagnosed with dysentery submitted a watery or bloody stool. However, the average lag between admission and specimen collection for these participants was 1.7 days and 43.8% (7/16) were treated with antibiotics prior to specimen collection potentially improving their condition before stool collection.

For participants diagnosed with diarrhoea with severe dehydration, 92% (11/12) were positive for at least one of *Campylobacter*, *Salmonella* or diarrhoeagenic *E. coli*. The remaining participant was negative for all pathogens, but was admitted three days prior to the specimen being taken and was treated with a combination of ampicillin and gentamicin, so potentially had cleared an infection prior to testing. The DEC pathotype *Shigella*/EIEC was detected in 3/4 specimens from participants diagnosed with dysentery, likely indicating *Shigella* associated bacillary dysentery and the other specimen was positive for the DEC pathotypes EAEC, EPEC and ETEC.

Pathogens

Of the 37 participants positive for *Campylobacte*r (Table 7), all but one were also positive for at least one other pathogen and 76% (28/37) were positive for 3-5 pathogen types in total. The majority (86%, 32/37) of co-infections were with diarrhoeagenic *E. coli*.

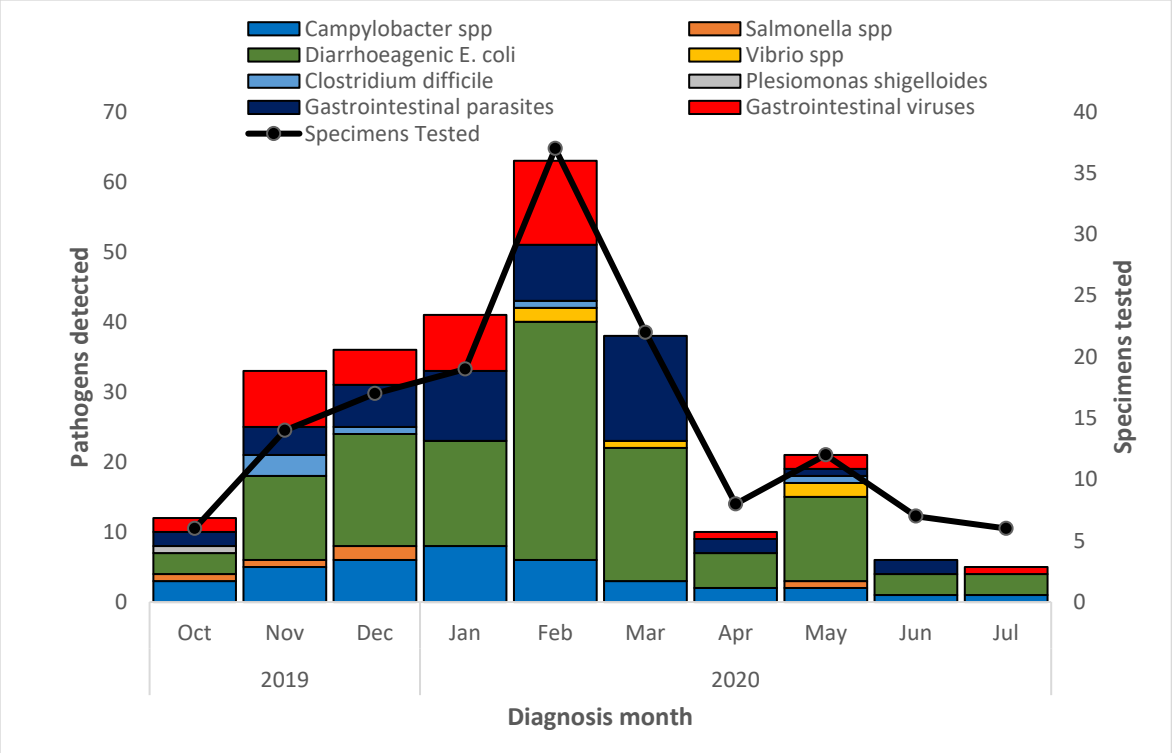
Five participants (3.4%, 5/148) had *Salmonella* detected in their specimen. The vast majority of all detections were for diarrhoeagenic *E. coli* strains, with at least one strain detected for 82% (122/148) of participants. Within these, there were 30 detections of *Shigella*/EIEC and four of STEC. Of these, only three detections of *Shigella*/EIEC were for participants who presented with dysentery.

There were five detections of *Vibrio* species, including three of *Vibrio cholerae* (noting the multiplex PCR is unable to determine if the strain is toxigenic). Dili is proximal to warm coastal waters, the natural habitat for *Vibrio* species. Both toxigenic and non-toxigenic *Vibrio* strains can produce severe watery diarrhoea, but the specimens of all 5 participants were not indicative of diarrhoea and their specimens were all positive for at least one other pathogen.

Parasite infections, which were rare in the birth cohort (4 detections in 141 specimens), were much more prevalent in the older participants (median 1.3 years) of the hospital enhanced surveillance, with 50 detections of at least one parasite including: 34 of giardia; 18 of cryptosporidium; and 12 of *Cyclospora cayetanensis*, respectively. Interestingly there were six co-infections of *Giardia* and *Cryptosporidium* and eight co-infections of *Giardia* and *Cyclospora cayetanensis*. The increased incidence of parasite infections in this cohort likely reflects higher exposure of older children to contaminated water either through drinking or recreational exposure, zoonotic transmission via animal contact, or person-to-person transmission.

Seasonality

Figure 6: Detections of gastrointestinal pathogens by multiplex PCR in the HNGV hospital enhanced surveillance vs specimens tested, October 2019 to July 2020



Noting that we have no results for the dry season months of August and September, and testing in the months of April to June was reduced due to the COVID-19 emergency period, we did identify seasonality for pathogen detections in the hospital enhanced surveillance (Figure 6, Table 9). We detected enteropathogens more commonly in the wettest months (December to March) and the highest ratio of pathogen detections to samples tested (2.3:1) occurred in December. However statistically significant seasonality was only identified for parasitic pathogens (OR 2.8, 95%CI 1.1-7.2, p=0.02). While detections for DEC as a group were not statistically significantly more frequent in the wet season, detections for individual pathotypes were: ETEC (OR 7.1, 95%CI 2.3-28.8, p<0.05); and EAEC (OR 2.4 95%CI 1.1-5.2, p<0.05). All four STEC and the two *E. coli* O157 detections were found in the wet season, but odds ratios could not be calculated due to zero cells.

Table 9: Number of pathogens detected* by multiplex PCR and percentage positive in the hospital enhanced surveillance by season

Pathogen	Wet Season (n=102)	Dry Season (n=46)	Odds Ratio (95% CI)	*p* value†
Parasites**	41 (40.2) §	9 (19.6)	2.8 (1.1- 7.2)	0.02
Clostridium difficile	2 (2.0)	4 (8.7)	0.2 (0.0-1.6)	0.08
DEC	88 (86.3)	34 (73.9)	2.2 (0.8-5.7)	0.10
Salmonella spp.	2 (2.0)	3 (6.5)	0.3 (0.0-2.6)	0.17
Plesiomonas shigelloides	0 (0.0)	1 (2.2)	n/a	0.31
Vibrio *spp.*	3 (2.9)	2 (4.3)	0.7 (0.1-8.3)	0.65
Campylobacter spp.	25 (24.5)	12 (26.1)	0.9 (0.4-2.3)	0.84
Viruses††	26 (25.5)	13 (28.3)	0.9 (0.4-2.1)	0.84
Yersinia enterocolitica	0 (0.0)	0 (0.0)	n/a	n/a

*Multiple pathogens can be detected per specimen; †Fisher's exact 2 tailed; §results reported as number positive (percent positive); **_Cryptosporidium_ spp., _Cyclospora cayetanensis, Giardia lamblia;_ DEC – Diarrhoeagenic _Escherichia coli;_ ††adenovirus F 40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus.

Isolation of bacterial enteropathogens

Overall in the hospital enhanced surveillance, *Campylobacter* and *Salmonella* were isolated twice each (Table 10). Comparing PCR and culture, for one specimen *Campylobacter* was positive on both PCR and culture and the other was PCR negative and culture positive. Both specimens were from children with a diagnosis of severe acute malnutrition. Two specimens were positive for *Salmonella* on both PCR and culture (one of which was also PCR positive for *Campylobacter*). Again both specimens were from children with a diagnosis of severe acute malnutrition.

Table 10: Detection of gastrointestinal pathogens* by multiplex PCR and culture, by percentage positive, in the hospital enhanced surveillance (n=148)

Pathogen	PCR+ (%)	Culture+ (%)
Campylobacter spp.	37 (25.0)	2 (1.4)
Salmonella spp.	5 (3.4)	2 (1.4)
DEC	122 (82.4)	0 (0.0)

*Multiple pathogens can be detected per specimen; DEC- *Diarrhoeagenic Escherichia coli*

Time lag

Many participants were admitted to HNGV, but a specimen was not submitted for testing for several days as recruitment relied on the patient being transferred to the paediatrics team, our study team to attend the next paediatrics morning rounds, and then to contact the parents or guardians of the patient to seek their consent for participation, before collecting a stool specimen. This did not affect the number of participants testing positive to multiple pathogens, likely reflecting the chronic nature of these infections and the high sensitivity of the PCR tests. Only one specimen was not tested in the laboratory on the day it was received, which is a great achievement for the NHL team.

7.2 Objective 2: Determining the potential contribution of social, zoonotic, and environmental factors in human enteropathogen infections in a small sample of infants in Timor-Leste.

At each home visit of the birth cohort, the parents or guardians were surveyed regarding social, environmental, food security and food safety issues.

7.2.1 Loss to follow up

Overall 28 (47%) birth cohort participants did not complete all four home visits. This comprised 12 participants who moved with their families out of the study area, 10 who became uncontactable, five who were withdrawn by a parent or guardian, and one participant who unfortunately died. It was discovered by our research team over the course of the study that the families of the birth cohort participants were very mobile. As HNGV is the major referral hospital for the country, many families move into Dili from other districts just prior to giving birth, stay with family and then move back home soon after the baby is born. Families also move in and out of Dili to access health care and pursue jobs, visit family and attend religious festivals. The COVID-19 emergency period also prompted many participant's families to move to join their extended families in villages in other regions of Timor-Leste. In future, revising recruitment strategies to take into account a mobile population including requesting alternate home addresses and factoring in greater travel times for specimen collection could reduce this attrition.

7.2.2 Household size

Household size varied greatly across birth cohort participants and even between home visits for individual participants. The fluctuation in household size was also a principal factor in the high level of loss-to-follow-up. Table 11 shows the breakdown of age groups over the whole study period.

Table 11: Household size for birth cohort families by age group

Age Group	Median (range)
0-4 years	2 (1-6)
5-17 years	1.75 (0-8)
18-64 years	4 (1-10)
65+ years	0 (0-2)
Overall	8 (3-15)

Households tended to be large with a median of eight people per household and range of 3-15 across the four home visits. There was also a median of four adults aged 18-64 per household, but as many as eight. In future research it would be useful to collect data on numbers of bedrooms and relationships of members of households as crowding and mobility of people in and out of households are factors in the spread of foodborne disease.

7.2.3 Family income and maternal education levels

The home visit questionnaire was completed by several different responsible adults and for two participants by a different responsible adult at each visit, however the participant's mother was by far the most common respondent, and answered at least once for all but two participants where the father answered each time. Of these 47 mothers: 23% (11/47) completed a tertiary qualification; 45% (21/47) completed secundario (12 years of education); 19% (9/47) started but did not complete secundario; 9% (4/47) attended primary school; and 4% (2/47) never attended school.

Just over half of the mothers with a tertiary education (55%, 6/11) reported being employed. A further two of the mothers who completed secundario (10%, 2/21) also reported being employed. The remaining mothers all reported doing housework (30) or being unemployed (9).

The question on household income was sensitive: 38% (18/47) of mothers refused to answer; and 45% (21/47) reported that they did not know. No respondents reported their family income in visit two or visit three and only one respondent reported their family income in visit four. Of those mothers that reported family income in the previous year, six reported an income of USD$730-$1460 and four reported USD$365-$730. Half of the mothers that reported their family income also reported being employed (5/10).

7.2.4 Nutrition and food safety

All survey respondents reported that cooking for their families was conducted inside, either within the house or in a separate building, and most families had a designated kitchen. The data collected from the birth cohort showed an early adoption of supplementary feeding practices, with 37% (18/49) of infants in the birth cohort being formula fed (2/49) or receiving supplementary formula feeds (16/49). These findings and discussions with the paediatric team at HNGV led to development of an addition to the study of a more detailed questionnaire about usage and mixing of formula which was implemented at visit 3. These questions showed that, despite seven different brands being used (none with instructions provided in Tetum), all 15 respondents believed they understood the mixing instructions. Of these respondents 80% (12/15) reported using the scoop, cup, or sachet provided with the product to measure. However, 47% (7/15) of respondents reported that they added water to formula rather than the correct method of adding formula to the water. Additionally, while the majority of respondents reported boiling bottles between uses at visit 1 and 2, this notably declined for visits 3 and 4 allowing the potential for contamination. Further investigation into the low exclusive breastfeeding rates and the challenges in provision of formula feeding will be important to address these issues affecting growth and nutrition.

7.2.5 Water, sanitation, and hygiene practices

Household garbage disposal varied from visit to visit, potentially reflective of seasonal change. At the first visit 65% (32/49) of respondents reported disposing via a garbage bin or dumpster and 29% (14/32) reported burning rubbish. Reported use of garbage bins or dumpsters increased in visit 2, and in visit 3 all 40 respondents reported using a garbage bin or dumpster. However at visit 4, only 53% (17/32) of respondents reported use of a garbage bin or dumpster, with 41% (13/32) reporting throwing rubbish away, probably reflective of a disruption in municipal garbage collection due to the pandemic. Garbage disposal is an ongoing concern in Dili. A recently published study reports that the local

district government maintains 335 city garbage bins and a fleet of eight waste disposal trucks and rents up to 30 private trucks for garbage disposal, but due to resource issues a significant portion of waste is not collected (23).

All respondents reported some version of a pit latrine, reflecting Dili's current lack of a municipal sewerage system. Dili regularly experiences flooding events during the Wet Season from December to April and on 13 March 2020 Dili experienced a major flooding event which destroyed roads and bridges, an estimated 200 homes and the NHL was also inundated[3]. These flooding events combined with ubiquitous pit latrines provide ample opportunities for transmission of enteric pathogens.

Household water sources changed from visit to visit during the study with an increasing dependence on bottled water indicating instability of sources and an over-reliance on uncovered water storage. All families did, however, demonstrate awareness and practice of water disinfection by boiling or bleach on at least one visit, with disinfection practices declining in visit 3 and 4 with the concomitant rise of sourcing bottled water for drinking. Water availability is an ongoing concern in Dili and only approximately 50% of all households are connected to mains water (24). More than half (53%, 26/49) of participant's families sourced at least some of their drinking water from a bore, well, or spring for at least one home visit and it was the principal source of drinking water for 24% (12/49) families at the first visit, 20% (8/41) at the second visit and only one family each at the third and fourth home visits.

Animal ownership

Household animals kept within the home/compound were common; every family that answered the question had at least one animal. However, this was seen as a sensitive topic that many participant families were either unable to or declined to answer. For the 41 families that did respond for at least one home visit, all reported owning at least one animal, with a median of 3.5 (range 1-18), over the four visits.

Most families that responded (61%, 25/41) reported at least one dog, median 1 (range 0-5), whereas cats were rare with only two families reporting them, although one of those families reported eight cats at one visit. Dogs and cats tended to roam free inside and outside the house. Pig ownership was common with 63% (26/41) of families reporting owning at least one pig, median 2 (range 0-5). Goats were much less common with only five families (12%) reporting owning at least one, median 1.5 (range 0-3). Most pigs and goats were kept tied or in pens or cages. No cow ownership was reported by respondents. Chickens were reported by 76% (31/41) respondents and total numbers fluctuated substantially over the four visits, median 3 (range 0-15[4]). The majority of chickens roamed free in the yard. The ubiquitous presence and free movement of chickens represents a risk for transmission of enteric pathogens, particularly *Campylobacter*. Of participants who were positive for *Campylobacter* at least once during the four home visits, 85% (11/13) owned chickens; however, this was not a statistically significant association due to small numbers: OR 2.6 95%CI (0.4-28.8) p=0.27. While potentially representing a risk of enteric infection, chicken ownership also presents an opportunity for encouraging protein consumption in young children.

[3] *https://floodlist.com/asia/timor-leste-flooding-dili-march-2020*

[4] *Researchers stopped counting at 15 on this visit*

7.3 Objective 3: Building capacity in human and animal public health laboratory diagnosis, surveillance, and outbreak detection and investigation for enteropathogens in Timor-Leste.

7.3.1 Strong Partnerships

To facilitate linkages with Timor-Leste institutions, project leaders met with local staff at the National Health Laboratory (NHL), Diagnostics Laboratory for Animal Health (DLAH), Hospital Nacional Guido Valadares (HNGV), Institute Nacional Saude (INS), and WHO Communicable Disease Control (CDC), on an initial scoping visit.

A second visit from 18-20 March 2019 involved a presentation to the Institute National of Health-Research Ethics & Technical Committee to receive ethics approval for the study. Ethics approval was received and subsequently also provided by ANU. More collaboration meetings were held with partners, and training was provided for NHL and DLAH staff in isolating *Campylobacter* species.

From the beginning of this project, the level of support provided by potential partners was extremely high. The Ministry of Health and INS provided considerable advice and support in creating the project and the HREC provided positive feedback which improved the project. The Ministry of Agriculture and Fisheries (MAF) and the DLAH were also eager to collaborate and the joint laboratory training session on *Campylobacter* isolation at the NHL was a great success.

In July 2019 we also met with the ACIAR funded TOMAK (To'os ba Moris Di'ak/Farming for Prosperity) team to discuss mutual interests in food security which has led to a new joint proposal to ACIAR for future collaborative research.

Members of the DFAT funded STRONG-TL project (Menzies School of Health Research) were also members of this study team and we collaborated closely with them from the start of this study. Menzies generously shared their in-country resources and expertise and worked closely with our research team. When ANU staff could no longer travel to Timor-Leste due to COVID-19 travel restrictions, the Menzies team took an even more active role in assisting our research team to complete the study.

In addition to the findings reported here, the strength of the collaboration with the HNGV paediatric team led to the development of additional research. Our study detected a high level of infant formula use at even one month of age. Upon discussing these results with the paediatrics team, they raised a concern that over-dilution and under-dilution of infant formula is occurring due to lack of standardisation and few formula packets including instructions written in Tetum. A study will be undertaken in collaboration with Menzies to further investigate food safety aspects related to infant formula when the COVID-19 restrictions permit.

7.3.2 Research Team

In July 2019, the research team recruited and trained two local staff in Timor-Leste to manage the project: a full-time research manager and a full-time research nurse to manage recruitment for the cohort study, conduct field testing, collect specimens, and conduct simple data analysis.

Over a two-week period, the team attended face to face training sessions in Good Clinical Practice for research (GCP) including confidentiality, undertaking participant recruitment, obtaining informed consent, interview techniques, data collection, recording, monitoring and reporting (delivered by the ANU researchers). In addition, the key staff were trained in REDCap data software for collecting, and securely storing the project records, protocols for accurately measuring and weighing infants, and instructing parents/ guardians on the

safe collection of stool specimens. Standard operating procedures were developed for all aspects of this research and staff were trained in their use.

The family visits progressed very well; the 2-person team of one male and one female was well received by families and no major issues arose. The sling baby scale became difficult to use as the infants grew, and a larger calibrated portable baby scale was provided. As a stool sample was not always available for collection on the day of the home visit, several return visits were required to collect stool specimens. Home visit 3 was underway in March 2020 when our research manager Almerio Moniz left the project for a permanent position. We rapidly recruited a new research manager Antoniho Gusmao. He was provided with a limited handover by Almerio, as the Project team from ANU were unable to travel to Dili to complete comprehensive training due to the COVID-19 travel restrictions. Over the first month of the new manager's employment with the team, the ANU team met with him at least weekly to provide clarifications and support. Throughout the study, the small research team were provided day to day support by the STRONG TL team, particularly Dr Nick Fancourt, Nevio Sarmento and Paulino da Silva (Pulsa study nurse) as well as the paediatric team at HNGV and the laboratory team and NHL.

On 23 occasions across the home visits, the research team had to return to a birth cohort participant's home after a home visit as a stool specimen was not available at the time of the original visit. This was not a concern for this study but may be an issue for scaling up over a larger geographical area. Collecting stool specimens is also an imposition on busy families, easily forgotten, and requires training in safe handling and provision of safety equipment. Specimens collected in this manner have varied duration between collection and testing, potentially affecting pathogen yield.

In future, it may be beneficial to have appropriately trained research staff use flocked rectal swabs to safely collect specimens during the home visit. A study in children in Botswana showed no significant difference in pathogen detection rates by the BioFire system between flocked rectal swab samples and traditional stool sampling methods (25). Rectal swabs have traditionally provided lower yield to stool specimens for bacterial culture, however some recent studies in children (26) and adults (27) have shown relatively high success with culture from rectal swabs. Culture from stool specimens remains the gold standard, but the use of rectal swabs could bring significant convenience benefits, particularly in studies spread over a larger geographical area.

At the commencement of the study, Dr Flavio Brandão of HNGV generously agreed to provide office space at the hospital for the research team rent free. Initially, the team were graciously accommodated with the St John of God Midwifery team, who also facilitated introductions to maternity ward and obstetric staff. This was beneficial to the development and initiation of the study, and gave the research team valuable support in learning HNGV protocols and developing networks that supported them throughout the study.

The rapid progress of major physical upgrades to HNGV over the course of 2019 warranted two office moves by the research team within HNGV and co-location with the STRONG TL PULSA research team, when demand for hospital office space was at a premium. When the Menzies School of Health Research established an office in central Dili, they provided the research team accommodation for the remainder of the study.

7.3.3 Education and Training

The pilot project contributed to enhanced capacity in the local laboratory and health research workforce and their support also greatly assisted in the development of our own research team. We developed strong linkages with HNGV, particularly with the wonderful paediatrics department who graciously allowed our research team to attend daily rounds meetings to enhance their learning and development and to identify children aged 0-9 admitted with severe acute malnutrition, diarrhoea with severe dehydration or dysentery symptoms for the hospital arm of our study. We also greatly appreciated the advice and

assistance received from the St John of God and HNGV maternity teams in recruiting the birth cohort, Timor-Leste Ministries of Health and Agriculture and Fisheries and Maluk Timor. Our STRONG-TL partners greatly assisted our research program by partnering with our research team to enhance learning and development and streamline participant recruitment, via expert advice throughout the project, and even assisting with providing an office for our team when the original became unavailable due to renovations.

Our team reciprocated: Dr Samantha Colquhoun travelled to Dili for an additional two weeks (funded through the STRONG-TL program) to work with the research team and to facilitate provision the first two modules of applied operational research training (SORT-IT course) to 19 local health professionals from Ministry of Health (MoH), World Health Organization (WHO), HNGV, NHL, in collaboration with STRONG-TL, the Timor-Leste MoH, Maluk Timor, INS and other collaborators from Australia and Timor-Leste. Our research manager, Almerio Moniz, and the NHL team leader Virginia da Conceicao both completed this training and developed their own research projects. While Almerio was unable to see the whole project through, his designed project passed ethics review, was added to ours and enhanced results. Dr Samantha Colquhoun also supervised a surveillance officer from the MoH to complete his research project which focuses on improving syndromic disease surveillance in Timor-Leste.

Training was reinforced and feedback sought to address areas that required more support or input from the Australian investigators. Field site visits were undertaken by BP and SC in July, August, and October 2019 and in January 2020 to provide face to face training, monitor data and liaise with the co-investigators and collaborating organisations. Further planned travel was cancelled due to pandemic travel restrictions, remote support continued throughout the period of the study.

7.3.4 National Health Laboratory capacity

To build capacity among local partners, initial training was provided to paediatric and maternity staff of HNGV on the objectives of the study and the recruitment process in March 2019. The laboratory staff from the National Health Laboratory, Diagnostics Laboratory for Animal Health and the Ministry of Health attended a training session on culture of enteric pathogens in July 2019. The laboratory staff at the National Health Laboratory have had continuous on-ground support from Australian laboratory scientists and microbiologists throughout 2019-20 with the STRONG-TL and Fleming fund projects that aim to increase all aspects of laboratory functions. NHL staff received significant training and development opportunities via greater specimen throughput in the laboratory. Over the course of the study, 292 specimens were tested.

7.3.5 Diagnostics Laboratory for Animal Health Laboratory Capacity

DLAH staff also attended the training session on culture of enteric pathogens in July 2019. Three home visits were performed by DLAH staff in collaboration with the research team to the homes of birth cohort participants who were positive for enteric pathogens after their first home visit. These were successful, with the research team and the DLAH team attending together, sampling from fresh faeces and directly from animals and then testing the results as per *7.1 Animal and environmental testing*. However due to the detection of African Swine Fever in Timor-Leste in September 2019, the DLAH were unable to spare resources to continue these visits. Despite this the collaboration remained strong.

7.3.6 Restocking Laboratory Disposables

The difficulties of procuring laboratory supplies within Timor-Leste are well known and the project plan factored this into the design, with all study supplies being purchased in Australia and transported to Dili as luggage by Australian members of the research team when traveling for site visits and via freight with Menzies School of Health shipments during the time of the COVID-19 pandemic. These methods proved successful but were

problematic. In early 2020, the bushfires around Canberra delayed a shipment of supplies and later the COVID-19 pandemic reduced travel between the countries. The majority of disposables had been transported by this time, but poured agar plates have a relatively short shelf-life, so disruption of travel plans meant that the NHL ran out of *Campylobacter* selective agar plates before the end of the final home visit. Agar plates are heat-sensitive so flight delays in combination with hot temperatures could render plates unusable. No significant flight delays occurred during the course of the study.

In future, providing bulk agar mix so that the NHL and DLAH can pour and mix their own plates rather than providing pre-poured plates would be preferable. It is a more expensive option (approximately triple before factoring in transport costs) but has a long shelf life and is a more reliable method of maintaining supply. Preparing agar in the lab is not commonly done in Australia anymore as there is an economy of scale in purchasing pre-poured plates from large suppliers, but it is a critical skill in low- and middle-income countries and there are laboratory scientists at the NHL and DLAH that are skilled in this practice.

7.3.7 Study Limitations

As a small pilot study, our study is underpowered to demonstrate strong associations. Rather our focus was on building stronger links with human and animal health in Timor-Leste and local capacity in public health research, human and animal public health laboratory diagnosis and surveillance as well as identifying the areas to focus on in future research and the existing barriers to that research that will need to be mitigated.

Several deviations from the original plan occurred due to complications from the COVID-19 pandemic, African Swine Fever Outbreak, and difficulty in isolating pathogens on selective media. The original plan for the birth cohort was to have the home visits in the 1st, 3rd, 5th, and 7th months of life, but a no-cost extension was approved, and the home visits were adjusted to be approximately in the 1st, 3rd, 5th, and 12th months of life.

Our pathogen testing data must be interpreted with caution, as we predominantly recorded positive results via multiplex PCR testing, which is very sensitive and detects presence of pathogen nucleic acid but not necessarily viable pathogen. However, similar studies have shown that multiplex PCR testing can provide an accurate picture of enteropathogen burden in low- and middle-income countries (28, 29).

Campylobacter is a fastidious organism requiring a narrow range of temperatures (37-42^{0}C) and microaerophilic conditions for culturing (30, 31). It was known at the outset of the study that the NHL did not have access to a 42^{0}C incubator. Therefore, expert microbiologist input was sought, and a plan was developed to isolate *Campylobacter* species at 37^{0}C in Dili. *Campylobacter* strains were streaked onto *Campylobacter* blood-free selective agar plates (ThermoFisher Australia) and incubated at 37^{0}C for 48hr in an Oxoid 2.5 L anaerobe jar. A microaerophilic atmosphere was provided by the use of CampyGen sachets (Oxoid, Australia). The agar plates were chosen as they are designed to be selective for *Campylobacter* species at 37^{0}C. Despite these preparations, we had minimal success isolating *Campylobacter*. This could be due to several factors, including process deficits; however, the laboratory had expert oversight while learning and conducting this procedure. Therefore, possible explanations include that, particularly for the birth cohort, the much higher sensitivity of PCR testing may have been detecting dead pathogens or live pathogens at levels too low to be cultured. Another possibility is that the species of *Campylobacter* causing enteric infections in Dili are not the common species detected in Australia i.e. *C. jejuni* and *C. coli*.

African Swine Fever was detected in Timor-Leste in September 2019, representing a major animal health emergency. The DLAH were then understandably unable to spare resources to continue home visits. However the first three visits gave support to our hypothesis that exposures around the home environment were potential sources of enteropathogen infection for our birth cohort.

At the end of the Project, a workshop was planned to disseminate our findings to relevant government and non-government agencies and the OneHealth study community. Due to the COVID-19 situation in Timor-Leste, this workshop has not been held. Options to hold this workshop virtually were explored, but internet stability is a significant issue in Timor-Leste and the pressing nature of the COVID-19 response made it unlikely that several major collaborators would be able to spare the time to participate.

8 Conclusions and recommendations

8.1 Conclusions

Although malnutrition is multi-factorial, we have demonstrated that foodborne disease is an important but overlooked component of the full picture of malnutrition in Timor-Leste. We have documented the real impact of enteric disease on children in Dili, easily recruiting 150 children admitted to a single paediatric unit for treatment of diarrhoea and severe dehydration, dysentery, and predominantly severe acute malnutrition over a 10-month period. Nine in ten of these children were positive for at least one enteropathogen and almost six in ten were positive for multiple pathogens. In the birth cohort, almost three quarters of specimens from predominantly asymptomatic infants were positive for an enteropathogen.

We have trained and developed a small local team of health researchers who have conducted our study, developed strong links with the STRONG-TL research team and gone on to develop their own research as mentioned above.

At the outset of this project, we met with our partners and learned that while the NHL and DLAH had good facilities and expert staff, they had not the materials or experience in detecting and isolating a fastidious organism like *Campylobacter*. *Campylobacter* is the most commonly reported cause of bacterial enteric disease in the world and has been associated with malnutrition in several large studies so was the main pathogen focus of study. We provided the materials, assisted in the training and most importantly provided the specimen throughput to detect and culture *Campylobacter* at both NHL and DLAH. While overall success in isolating Campylobacter was minimal both the NHL and DLAH did succeed in doing so and we have learnt much about the best ways to support their efforts.

We have experienced, in one single year, the myriad challenges that are faced by human and animal health in Dili, and the people that they serve. We have also witnessed their resilience in meeting these challenges. This study was affected by both animal and human pandemics in African Swine Fever and COVID-19 and natural disaster in the form of the devastating flood of March 2020. We have emerged with strong partners that are keen to continue working together to further elucidate the linkages between foodborne bacterial enteropathies and malnutrition in Timor-Leste.

8.2 Recommendations

Given the critical role that poultry plays in the lives of families, we recommend a further study to explore infant and child dietary practices, food safety and environmental hygiene conditions in Timor-Leste. The study should focus on community poultry production and potential risks of *Campylobacter,* diarrhoeagenic *Escherichia coli,* and *Salmonella* and consider targeted interventions in urban and rural settings in Timor-Leste, utilising a One Health collaborative approach.

Our results clearly show the infection risks outside the home but do not explore risks inside the home in similar detail. Exploring the food safety aspects of food preparation and storage and collecting and testing environmental specimens from inside the home would be a logical next step for study.

Early adoption of supplementary feeding was very evident in this study. It is a known issue in Timor-Leste, and HNGV in particular has strong programs that promote exclusive breast feeding to six months of age. Providing culturally appropriate advice that complements and directs families towards existing programs is recommended and will require further investigation.

Animal ownership was identified as both critical and sensitive. Providing culturally sensitive advice about building fenced runs for chickens could both reduce human enteropathogen infection risk and provide protection for the birds from predation.

Rainwater storage tanks were commonly reported by our study participants with high numbers uncovered at least some of the time. Uncovered water tanks can be contaminated by rodents or birds, but even covered rainwater tanks can pick up pathogens and pollutants via the roof collection. Additionally, all participant families report use of a pit latrine and over half of families source some or all of their drinking water from ground sources such as wells and bores. These, in combination with frequent flooding, can lead to the proliferation of enteropathogen infections. Both our birth cohort and the enhanced laboratory surveillance identified that enteropathogen detection is more common in the wet season.

Culturally sensitive recommendations to ensure water storage is covered, to treat all rainwater and groundwater prior to drinking, and how to avoid disease after flooding events would be beneficial.

In liaison with local partners, we recommend preparing locally relevant information on safe food preparation for families involved in the pilot study and for use in HNGV.

A final summary report based on this report (included at 10.2) will be translated into Tetum and disseminated among project partners and the Ministries of Health and Agriculture and Fisheries.

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10 Appendices

10.1 Supplementary Tables

Table S1 Number of pathogens detected* by multiplex PCR and percentage positive in the birth cohort at each home visit.

Pathogen	Visit 1 (n=45)	Visit 2 (n=34)	Visit 3 (n=36)	Visit 4 (n=26)	*p* value†
Campylobacter spp.	3 (6.7)§	1 (2.9)	3 (8.3)	8 (30.8)	<0.01
Enteroaggregative *E. coli*	11 (24.4)	21 (61.8)	30 (83.3)	15 (57.7)	<0.01
Enteropathogenic *E. coli*	3 (6.7)	10 (29.4)	21 (58.3)	15 (58.7)	<0.01
Enterotoxigenic *E. coli*	1 (2.2)	4 (11.8)	12 (33.3)	5 (19.2)	<0.01
Cryptosporidium	0 (0.0)	0 (0.0)	0 (0.0)	3 (11.5)	<0.01
Adenovirus F40/41	0 (0.0)	0 (0.0)	2 (56)	4 (15.4)	<0.01
Rotavirus A	0 (0.0)	3 (8.8)	2 (5.6)	4 (15.4)	0.02
Shiga-toxigenic *E. coli*	0 (0.0)	0 (0.0)	0 (0.0)	2 (7.7)	0.03
Norovirus GI/GII	0 (0.0)	3 (8.8)	7 (19.4)	2 (7.7)	0.04
Clostridium difficile (toxin A/B)	1 (2.2)	4 (11.8	4 (11.1)	4 (15.4)	0.06
Sapovirus (I, II, IV, and V)	0 (0.0)	0 (0.0)	4 (11.1)	0 (0.0)	0.20
Salmonella spp.	0 (0.0)	0 (0.0)	4 (11.1)	0 (0.0)	0.20
Vibrio cholerae	0 (0.0)	0 (0.0)	2 (5.6)	0 (0.0)	0.37
Plesiomonas shigelloides	0 (0.0)	0 (0.0)	1 (2.8)	0 (0.0)	0.53
Giardia lamblia	0 (0.0)	0 (0.0)	1 (2.8)	0 (0.0)	0.53
Shigella/Enteroinvasive *E. coli*	1 (2.2)	0 (0.0)	1 (2.8)	0 (0.0)	0.69
Vibrio spp.	1 (2.2)	0 (0.0)	2 (5.6)	0 (0.0)	0.96
Cyclospora cayetanensis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	n/a
Astrovirus	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	n/a
E. coli O157	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	n/a
Yersinia enterocolitica	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	n/a

*Multiple pathogens can be detected per specimen; †χ^2 test for trend; §results reported as number positive (percent positive).

Table S2: Number of pathogens detected* by multiplex PCR and percentage positive in the birth cohort by season

Pathogen	Wet Season (n=52)	Dry Season (n=89)	Odds Ratio (95% CI)	*p* value†
Enteroaggregative *E. coli*	42 (80.1)§	35 (39.3)	6.5 (2.7-16.2)	<0.01
Enteropathogenic *E. coli*	29 (55.8)	20 (22.5)	4.4 (1.9-9.8)	<0.01
Enterotoxigenic *E. coli*	14 (26.9)	8 (9.0)	3.7 (1.3-11.1)	<0.01
Norovirus	10 (19.2)	2 (2.2)	10.4 (2.0-99.7)	<0.01
Salmonella spp.	4 (7.7)	0 (0.0)	n/a	0.02
Sapovirus (I, II, IV, and V)	4 (7.7)	0 (0.0)	n/a	0.02
Vibrio cholera	2 (3.8)	0 (0.0)	n/a	0.13
Campylobacter spp.	3 (5.8)	12 (13.5)	0.4 (0.1-1.6)	0.26
Cryptosporidium	0 (0.0)	3 (3.4)	0 (0.0-2.2)	0.30
Giardia lamblia	1 (1.9)	0 (0.0)	n/a	0.37
Plesiomonas shigelloides	1 (1.9)	0 (0.0)	n/a	0.37
Shiga-toxigenic *E. coli*	0 (0.0)	2 (2.2)	0 (0.0-3.3)	0.53
Vibrio spp.	2 (3.8)	1 (1.1)	3.5 (0.2-210.0)	0.55
Shigella/Enteroinvasive *E. coli*	1 (1.9)	1 (1.1)	1.7 (0.0-136.9)	1.00
Adenovirus F40/41	2 (3.8)	4 (4.5)	0.9 (0.1-6.2)	1.00
Rotavirus A	3 (5.8)	6 (6.7)	0.8 (0.1-4.2)	1.00
Clostridium difficile (toxin A/B)	5 (9.6)	8 (9.0)	1.1 (0.3-4.0)	1.00
E. coli O157	0 (0.0)	0 (0.0)	n/a	n/a
Cyclospora cayetanensis	0 (0.0)	0 (0.0)	n/a	n/a
Astrovirus	0 (0.0)	0 (0.0)	n/a	n/a
Yersinia enterocolitica	0 (0.0)	0 (0.0)	n/a	n/a

*Multiple pathogens can be detected per specimen; †Fisher's exact 2 tailed; §results reported as number positive (percent positive)

Table S3 No. of pathogens detected* by multiplex PCR and percentage positive in the hospital enhanced laboratory surveillance, by diagnosis

Pathogen	DWSD or dysentery (n=16)	SAM (n=132)	Odds Ratio (95% CI)	*p* value†
Shigella/Enteroinvasive *E. coli*	7 (43.8)§	23 (17.4)	3.7 (1.0-12.3)	0.02
Vibrio spp.	2 (12.5)	3 (2.3)	6.1 (0.5-57.3)	0.09
Vibrio cholerae	1 (6.3)	2 (1.5)	4.3 (0.1-86.5)	0.29
Cyclospora cayetanensis	0 (0.0)	12 (9.1)	0.0 (0.0-2.5)	0.36
Giardia lamblia	2 (12.5)	32 (24.2)	0.0 (0.1-2.1)	0.36
Adenovirus F40/41	1 (6.3)	3 (2.3)	2.9 (0.1-38.0)	0.40
Salmonella spp.	1 (6.3)	4 (3.0)	2.1 (0.0-23.4)	0.40
Rotavirus A	1 (6.3)	6 (4.6)	1.4 (0.0-12.8)	0.56
Astrovirus	1 (6.3)	6 (4.6)	1.4 (0.0-12.8)	0.56
Enteropathogenic *E. coli*	10 (62.5)	71 (53.8)	1.4 (0.4-5.1)	0.60
Cryptosporidium	1 (6.3)	17 (12.9)	0.5 (0.0-3.3)	0.69
Campylobacter spp.	3 (18.8)	34 (25.8)	0.7 (0.1-2.6)	0.76
Enterotoxigenic *E. coli*	4 (25.0)	41 (31.1)	0.7 (0.2-2.6)	0.78
Enteroaggregative *E. coli*	10 (62.5)	85 (64.4)	0.9 (0.3-3.3)	1.00
Clostridium difficile (toxin A/B)	0 (0.0)	6 (4.6)	0.0 (0.0-5.4)	1.00
E. coli O157	0 (0.0)	2 (1.5)	0.0 (0.0-16.6)	1.00
Shiga-toxigenic *E. coli*	0 (0.0)	4 (3.0)	0.0 (0.0-8.2)	1.00
Plesiomonas shigelloides	0 (0.0)	1 (0.8)	n/a	1.00
Sapovirus (I, II, IV, and V)	1 (6.3)	13 (9.9)	0.6 (0.0-4.7)	1.00
Norovirus GI/GII	1 (6.3)	13 (9.9)	0.6 (0.0-4. 7)	1.00
Yersinia enterocolitica	0 (0.0)	0 (0.0)	n/a	n/a

*Multiple pathogens can be detected per specimen; †Fisher's exact 2 tailed; §results reported as number positive (percent positive)

Table S4 No. of pathogens detected* by multiplex PCR and percentage positive in the hospital enhanced laboratory surveillance, by season

Pathogen	Wet Season (n=52)	Dry Season (n=89)	Odds Ratio (95% CI)	*p* value†
Enterotoxigenic *E. coli*	41 (40.2)§	4 (8.7)	7.1 (2.3-28.8)	<0.01
Enteroaggregative *E. coli*	72 (70.6)	23 (50.0)	2.4 (1.1-5.2)	0.03
Rotavirus A	2 (2.0)	5 (10.9)	0.2 (0.0-1.1)	0.03
Cryptosporidium	16 (15.7)	2 (4.4)	4.1 (0.9-38.0)	0.06
Giardia lamblia	28 (27.5)	6 (13.0)	2.5 (0.9-8.0)	0.06
Sapovirus (I, II, IV, and V)	13 (12.8)	1 (2.2)	6.6 (0.9-285.4)	0.07
Clostridium difficile (toxin A/B)	2 (2.0)	4 (8.7)	0.2 (0.0-1.6)	0.08
Cyclospora cayetanensis	11 (10.8)	1 (2.2)	5.4 (0.7-239.2)	0.11
Enteropathogenic *E. coli*	60 (58.8)	21 (45.7)	1.7 (0.8-3.7)	0.16
Salmonella spp.	2 (2.0)	3 (6.5)	0.3 (0.0-2.6)	0.17
Shigella/Enteroinvasive *E. coli*	24 (23.5)	6 (13.0)	2.1 (0.7-6.6)	0.19
Shiga-toxigenic *E. coli*	4 (3.9)	0 (0.0)	n/a	0.31
Plesiomonas shigelloides	0 (0.0)	1 (2.2)	n/a	0.31
Astrovirus	6 (5.9)	1 (2.2)	2.8 (0.3-132.2)	0.44
Vibrio cholerae	3 (2.9)	0 (0.0)	n/a	0.55
Adenovirus F40/41	2 (2.0)	2 (4.4)	0.4 (0.0-6.3)	0.59
Vibrio spp.	3 (2.9)	2 (4.4)	0.7 (0.1-8.3)	0.65
Campylobacter spp.	25 (24.5)	12 (26.1)	0.9 (0.4-2.3)	0.84
E. coli O157	2 (2.0)	0 (0.0)	n/a	1.00
Norovirus GI/GII	10 (9.8)	4 (8.7)	1.1 (0.3-5.3)	1.00
Yersinia enterocolitica	0 (0.0)	0 (0.0)	n/a	n/a

*Multiple pathogens can be detected per specimen; †Fisher's exact 2 tailed; §results reported as number positive (percent positive)

10.2 Summary Report

Background

The prevalence of malnutrition in children under five years of age in Timor-Leste has significantly decreased in the last decade but remains amongst the highest globally. Diarrhoea is the second most common disease identified in Timorese children. There is a gap in current knowledge on the prevalence of enteropathogen infections in Timor-Leste and their potential association with malnutrition in young children.

Methods

A cohort of babies born at Hospital Nacional Guido Valadares (HNGV) were recruited in July-September 2019. They were visited at home at approximately 1, 3, 5 and 12 months of age. At each visit, height and weight were measured, a stool specimen was collected and a parent or guardian answered a questionnaire on feeding practices, household size, water, sanitation, and hygiene (WASH) practices and accessibility, and household animal ownership and containment. Households were provided a USD$2.00 mobile phone recharge card at each visit.

Children aged 0-4 years admitted to HNGV with a diagnosis of severe acute malnutrition, diarrhoea with severe dehydration, or dysentery were recruited, and a stool specimen was collected. All stool specimens were tested at the National Health Laboratory using a BioFire FilmArray Gastrointestinal Panel polymerase chain reaction (PCR) assay and specimens positive by PCR for *Campylobacter, Salmonella* or *Shigella*/EIEC, were reflex cultured on selective media.

Birth cohort participants who were PCR positive for at least one of *Campylobacter, Salmonella* or *Shigella*/EIEC were offered a further home visit where a team from the Diagnostics Laboratory for Animal Health (DLAH) collected animal faecal specimens from home environment which were analysed by culture for *Campylobacter*, *Salmonella*, and *Shigella on* selective media at DLAH.

Data was cleaned in STATA 15.1. Descriptive analysis performed in R Studio. Anthropometric measures converted to Z scores and mapped using the WHO R package “anthro”. Fisher’s exact tests, χ^2 test for trend, and odds ratios were calculated when appropriate, and p <0.05 was considered significant for all tests. Ethics was approved through IN-HRETC ref: 462/MS-INS/GDE/IV/2019 and ANU ref: 2019/221.

Malnutrition was assessed by weight-for-length/height Z (WHZ) scores for the birth cohort and weight-for-age Z (WAZ) scores for the hospital enhanced surveillance. These were also mapped against the WHO child growth standards using the R package “anthro”.

Results

Birth cohort

Of 60 babies recruited, 47% (28/60) were female. Only 52% (32/60) were retained until the final visit. Participants at birth demonstrated a median WHZ of 0.90 (IQR -0.32 to -1.78). However the median WHZ dropped to -1.2 (IQR -1.8 to -0.6) at the first home visit and by the final home visit at approximately 12 months of age, the median WHZ was -0.94 (IQR -1.76 to 0.16).

69% (97/141) of specimens were PCR positive for at least one enteric pathogen. However, no birth cohort specimens were culture positive. The pathogen load detected in the birth cohort increased over time and this was statistically significant for *Campylobacter, Salmonella*, Diarrhoeagenic *Escherichia coli* (DEC) strains, parasites and viruses. Co-infection was also common. DEC strains were the most common pathogens detected, with 85 detections in 34 participants over the four visits. Most (91%, 31/34) participants had multiple DEC detections, and 18% (6/34) were positive for a DEC strain at all four visits. Most pathogen detections increased in the wet season (statistically

significant for DEC and viruses), but *Campylobacter* and parasite detections were more common (not significant) in the dry season (Table 1).

Table 1: No. of pathogens detected* by multiplex PCR and % positive in the birth cohort by season

Pathogen	Wet Season (n=52)	Dry Season (n=89)	OR (95% CI)	*p* value†
DEC	44 (84.6)§	41 (46.1)	6.4 (2.6-17.5)	<0.01
Viruses**	18 (34.6)	11 (12.4)	3.7 (1.5-9.7)	<0.01
Salmonella spp.	4 (7.7)	0 (0.0)	n/a	0.02
Vibrio spp.	3 (5.8)	1(1.1)	5.3 (0.4-285.5)	0.14
Campylobacter spp.	3 (5.8)	12 (13.5)	0.4 (0.1-1.6)	0.26
Plesiomonas shigelloides	1 (1.9)	0 (0.0)	n/a	0.37
Clostridium difficile	5 (9.6)	8 (9.0)	1.1 (0.3-4.0)	1.00
Parasites††	1 (1.9)	3 (3.4)	0.6 (0.0-7.2)	1.00
Yersinia enterocolitica	0 (0.0)	0 (0.0)	n/a	n/a

*Multiple pathogens can be detected per specimen; †Fisher's exact 2 tailed; DEC – Diarrhoeagenic *Escherichia coli;* §results reported as number positive (percent positive); **adenovirus F 40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus; ††*Cryptosporidium* spp.*, Cyclospora cayetanensis, Giardia lamblia.*

DLAH staff completed three home visits to participants who were PCR positive for *Campylobacter,* and *Campylobacter* was cultured from animal and/or environmental specimens for each. The outbreak of African Swine Fever in September 2019 prevented further home visits.

There was a median of 8 people per household (range 3-15), and a median of 4 adults aged 18-64 years per household (range 1-8). Questionnaires were answered by several different responsible adults, however the participant's mother answered at least once for 96% (47/49). Of these: 23% (11/47) completed a tertiary qualification; and 45% (21/47) completed secundario. Only mothers who completed tertiary education (55%, 6/11) and secundario (10%, 2/21) also reported being employed. Household income was rarely reported: 6 families reported an income in the previous year of USD$730-$1460; and 4 reported USD$365-$730.

At one month of age, 37% (18/49) of infants in the birth cohort were either formula fed (4%, 2/49) or receiving supplementary formula feeds (33%, 16/49). Additional questions were added to the questionnaire at visit 3 and these demonstrated that 80% (12/15) of respondents reported using the product provided measure for preparation. However, 47% (7/15) of respondents reported that they incorrectly added water to formula. While the majority of respondents originally reported boiling bottles between uses, this declined for visits 3 and 4.

Household water sources changed from visit to visit during the study with an increasing dependence on bottled water over the four visits. Half (53%, 26/49) of participant's families sourced at least some of their drinking water from a bore, well, or spring for at least one home visit and this was the principal source of drinking water for 24% (12/49) of families at the first visit, declining to 1 family each at visit 3 (3%, 1/40) and visit 4 (3%, 1/32). All households reported some version of a pit latrine.

Many families were unable or unwilling to respond to questions about animals, but of those who did respond at least once, 100% (41/41) reported owning at least one animal (median 3.5, range 1-18). Dogs (61%, 25/41, median 1, range 1-5), cats (5%, 2/41, range 1-8) and chickens (76%, 31/41, median 3, range 1-15) tended to roam free inside and outside the house; and pigs (63%, 26/41, median 2, range 1-5) and goats (12%, 5/41, median 1.5, range 1-3) tended to be kept tied, penned or caged.

Hospital enhanced laboratory surveillance

A sufficient stool specimen was collected and tested from 148/159 participants recruited through hospital enhanced surveillance. Of these 148 participants, 53% (79/148) were female (sex not recorded for 1 participant and 1 participant was aged 7 years). All 148 participants were severely stunted, exhibiting a median WAZ of -3.8 (IQR -4.8 to -3.1). The majority (89%, 132/148) were admitted with a diagnosis of severe acute malnutrition (SAM), 8% (12/148) with diarrhoea with severe dehydration (DWSD), and 3% (4/148) with dysentery. Pathogens were detected by PCR for 90% (133/148) of participants; two specimens each were culture positive for *Campylobacter* species and *Salmonella* species. However, 92% (11/12) of DWSD admissions were positive on PCR for at least one of *Campylobacter*, *Salmonella* or DEC (Table 2) and all dysentery admissions were positive for DEC, with 75% (3/4) positive for the individual DEC pathotype *Shigella*/EIEC. There was no statistically significant relationship between pathogen detection and diagnosis (Table 2) except the individual DEC pathotype *Shigella*/EIEC which was more commonly associated with DWSD/dysentery than SAM (OR 3.69 95%CI 1.0-12.3, $p<0.05$).

Table 2: No. of pathogens detected* by multiplex PCR and percentage positive in the hospital enhanced laboratory surveillance, by diagnosis

Pathogen	DSWD or dysentery (n=16)	SAM (n=132)	OR (95% CI)	*p* value†
Vibrio spp.	2 (12.5) §	3 (2.3)	6.14 (0.5-57.3)	0.09
Parasites **	3 (18.8)	47 (35.3)	0.4 (0.1-1.6)	0.26
DEC	15 (93.8)	107 (80.5)	3.5 (0.5-153.3)	0.31
Salmonella spp.	1 (6.3)	4 (3.0)	3.5 (0.0-23.4)	0.44
Campylobacter spp.	3 (18.8)	34 (25.6)	0.7 (0.1-2.6)	0.76
Viruses (%)††	5 (31.3)	34 (25.6)	1.3 (0.3-4.5)	0.76
Clostridium difficile	0 (0.0)	6 (4.6)	0.0 (0.0-5.4)	1.0
Plesiomonas shigelloides	0 (0.0)	1 (0.8)	n/a	1.0
Yersinia enterocolitica	0 (0.0)	0 (0.0)	n/a	n/a

*Multiple pathogens can be detected per specimen; DWSD–diarrhoea with severe dehydration, SAM–severe acute malnutrition; †Fisher's exact 2 tailed; §results reported as number positive (percent positive); DEC – Diarrhoeagenic *Escherichia coli;* ***Cryptosporidium* spp.*, Cyclospora cayetanensis, Giardia lamblia;* ††adenovirus F 40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus.

We detected enteropathogens more commonly in the wet season than the dry season and the highest ratio of detections to samples tested (2.3:1) occurred in December. This association was statistically significant for parasitic pathogens (OR 2.8, 95%CI 1.1-7.2, $p=0.02$), and for the individual DEC pathotypes ETEC (OR 7.1, 95%CI 2.3-28.8, $p<0.05$); and EAEC (OR 2.4 95%CI 1.1-5.2, $p<0.05$). All four STEC and the two *E. coli* O157

detections occurred during the Wet season but odds ratios could not be calculated due to zero cells.

Discussion

The birth cohort were normal for weight at birth but declined to the 25th percentile by 1 year. All of the participants in the hospital enhanced surveillance were severely stunted. Pathogen load was high and statistically significantly higher in the wet season for some pathogens. All of the birth cohort families used some form of pit latrine, more than half (53%) sourced at least some of their drinking water from a bore, well, or spring and there was an over-reliance on uncovered water storage. This, combined with high domestic animal ownership and frequent wet season flooding, provides ample opportunities for the transmission of enteropathogens.

Changes from the study plan occurred due to the COVID-19 pandemic, African Swine Fever Outbreak, and difficulty in isolating pathogens on selective media without a 42^{0}C incubator. The birth cohort home visits were to be held at 1, 3, 5, and 7 months, and were adjusted to 1, 3, 5, and 12 months. Pathogen testing data must be interpreted with caution, as multiplex PCR testing is very sensitive and detects presence of pathogen nucleic acid but not necessarily viable pathogen, meaning some of the detections in the birth cohort may be of infections that have already resolved.

Conclusions and Recommendations

Infants and children in Dili have a high enteric pathogen load and signs of growth retardation from the first months of life. Contributing factors are complex: enteric pathogens; WASH; domestic animals, household size, and early supplementary feeding, but this study was not able to measure their specific contributions.

Given the critical demonstrated role of poultry, we recommend a larger-scale urban and rural study to explore infant and child dietary practices, food safety and environmental hygiene conditions in relation to community poultry production with a focus on the risk of *Campylobacter,* DEC, and *Salmonella.* This project should consider targeted interventions in, utilising a One Health collaborative approach.