Norovirus Infections and Association with Animal Exposure in Sarawak, Malaysia

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of Master of Science
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ABSTRACT

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Abstract

Diarrheal diseases continue to be one of the most significant killers of children throughout the world, and many diarrheal diseases are zoonotic in nature. In Malaysia, not much is known about the viruses causing disease in humans and animals, and there is little evidence describing the role noroviruses play in diarrheal disease in the state of Sarawak. This study aimed to estimate the prevalence of noroviruses in children admitted to Sibu Hospital with acute diarrhea and the prevalence of norovirus in swine environments in Sarawak. We collected stool samples from children admitted to the hospital for acute gastroenteritis and by convenience sampling from registered pig farms. Stool samples were tested for norovirus genogroups I and II/IV. At the time of sample collection, information was collected about prior animal exposure and medical history including previous hospitalization for diarrhea. Of the 70 participants enrolled in the study, 3 tested positive for norovirus GII/IV. None of the swine stool samples tested positive for noroviruses. None of the animal exposure variables were statistically associated with increased odds of previous hospitalization for diarrhea, but prior cat exposure non-significantly increased the odds of previous hospitalization by 3.78 (95% CI 0.89, 16.11). Although norovirus is not highly prevalent in children in Sibu, Malaysia, diarrheal disease causes significant disease burden in the study population. Future work should aim to elucidate risk factors for severe diarrhea and to determine the prevalence of other disease-causing pathogens. This information will help clinicians better treat

their patients and public health officials design programs to minimize the risk exposure to prevent diarrheal disease.

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1. Introduction

1.1. Diarrheal Diseases

The Global Burden of Disease (GBD) study quantifies the morbidity and mortality of major diseases and their risk factors throughout the world.[1] Among the largest groups of diseases in the GBD is diarrheal disease. According to the GBD, in 2015 alone there were an estimated 2.39 billion cases of diarrhea, with 957.5 million cases in children under the age of five.[2] The number of deaths due to diarrheal diseases has decreased by 20.8% since 2005, but diarrhea still accounts for a large proportion of deaths worldwide.[2] In 2015 alone, diarrheal diseases were the 9th leading cause of death and were responsible for 1.31 million deaths.[2] In children under the age of five, diarrheal diseases are the 4th highest cause of death.[2] Fortunately, deaths due to diarrhea in children under the age of five has decreased in recent years, but the overall incidence of diarrheal disease in this age group has decreased more slowly.[2]

In 2015 the GBD sought to estimate the prevalence of 13 specific diarrheal pathogens. The three most common pathogens in children under 5 were rotavirus, *Cryptosporidium* spp., and *Shigella* spp. accounting for 29.3%, 12.1%, and 11.0% of disease in this population, respectively.[2] In addition to being one of the most prevalent pathogens in children under 5, rotavirus is the leading cause of death due to diarrhea.[2] In children aged 5-14, *V. cholerae* is the leading cause of death, while individuals 15 and older are most likely to die from diarrhea caused by *Shigella* spp.[2] However, these numbers are not consistent across country income level. *C. difficile* is the leading cause of

death from diarrhea in high income countries, while cholera infections are the most common cause in low income countries in Sub-Saharan Africa and Southeast Asia.[2]

There are a variety of risk factors for diarrheal infections. The two leading risk factors for diarrhea in 2015 were unsafe water and poor sanitation; these risk factors were responsible for 61.1 million disability-adjusted life years (DALYs) and 40.0 million DALYs, respectively.[2] Fortunately, these risk factors are modifiable. Increasing access to safe drinking water and use of improved sanitation are two of the most effective ways to prevent diarrheal disease.[3] Additionally, encouraging individuals to practice safe personal and food hygiene reduces risk of diarrheal disease.[3] Treating diarrheal diseases focuses on preventing dehydration and malnutrition by providing oral rehydration solution and intravenous fluids.[3]

1.2. Norovirus

Norovirus was first discovered in 1972 using electron microscopy following an outbreak of nonbacterial gastroenteritis in 1968 in Norwalk, Ohio.[4] Noroviruses are approximately 27nm in diameter and are members of the calciviridae family of viruses.[4,5] The viral genome is approximately 7500 bases of positive sense RNA.[6] Noroviruses are classified into five genogroups (GI-GV), with the distribution of genogroups varying in different parts of the world.[5]

Norovirus transmission is most frequent in colder months and occurs primarily through the fecal-oral route from contaminated food and water.[7,8] There is also limited evidence that norovirus can be transmitted via the airborne path when virus particles

become aerosolized during episodes of intense vomiting.[9] After exposure to the virus, symptoms typically appear in 24-48 hours and may include vomiting, acute diarrhea, and nausea.[6,7,10] On average, symptoms resolve within 1-3 days.[6,7,10]

Norovirus causes of a substantial burden of diarrheal disease throughout the world. A recent meta-analysis estimated that norovirus was responsible for approximately 18% of all cases of acute gastroenteritis, including diarrhea and vomiting, worldwide.[11] The overall prevalence of norovirus is lower among countries with higher overall all-cause mortality rates when compared to countries with lower rates.[11] Different genogroups cause disease in humans and pigs. Genogroups I, II, and IV commonly cause disease in humans, and some genotypes within genogroups I and II cause disease in pigs.[5,12-14] However, norovirus prevalence in pigs is not constant around the world. In North Carolina it is estimated that the prevalence of norovirus in finisher pigs is 18.9%, and in Japan the estimated prevalence is 17.5%.[13,15] Although nearly one in five pigs are infected with norovirus in North Carolina and Japan, estimates are much lower in Europe.[16] The burden of norovirus is highly variable around the world, and more research must be done in humans and pigs to better understand its ecology.

1.3. Sarawak, Malaysia

Sarawak is a Malaysian state covering more than 120,000 km^2 on the island of Borneo (Figure 1).[17] Sarawak is separated from peninsular Malaysia by the South China Sea.[17] The towns of Sibu and Kapit are both located on the Batang Rajang River, Malaysia's longest river, and travel between these two towns occurs primarily by boat.[17]

As of 2010, Sarawak's total population was 2.4 million people, with just over 299,000 people living in Sibu.[18] Sibu hospital is a public hospital located in the state of Sarawak, which opened in 1994.[19] It is the second largest hospital in Sarawak with 635 beds and serves as the referral hospital for central Sarawak.[19]

Diarrheal disease in Malaysia was responsible for an estimated 1.3 deaths per 100,000 in 2015 and resulted in 28,305.0 DALYs.[2] In children under 5, diarrheal disease led to 9952.2 DALYs and an estimated 2.01 deaths per 100,000 in 2015.[2] Since 2005, the mortality rate due to diarrheal disease in Malaysia decreased by 35.9%, but the number of DALYs only dropped 13.8%.[2] This suggests that fewer children are dying from

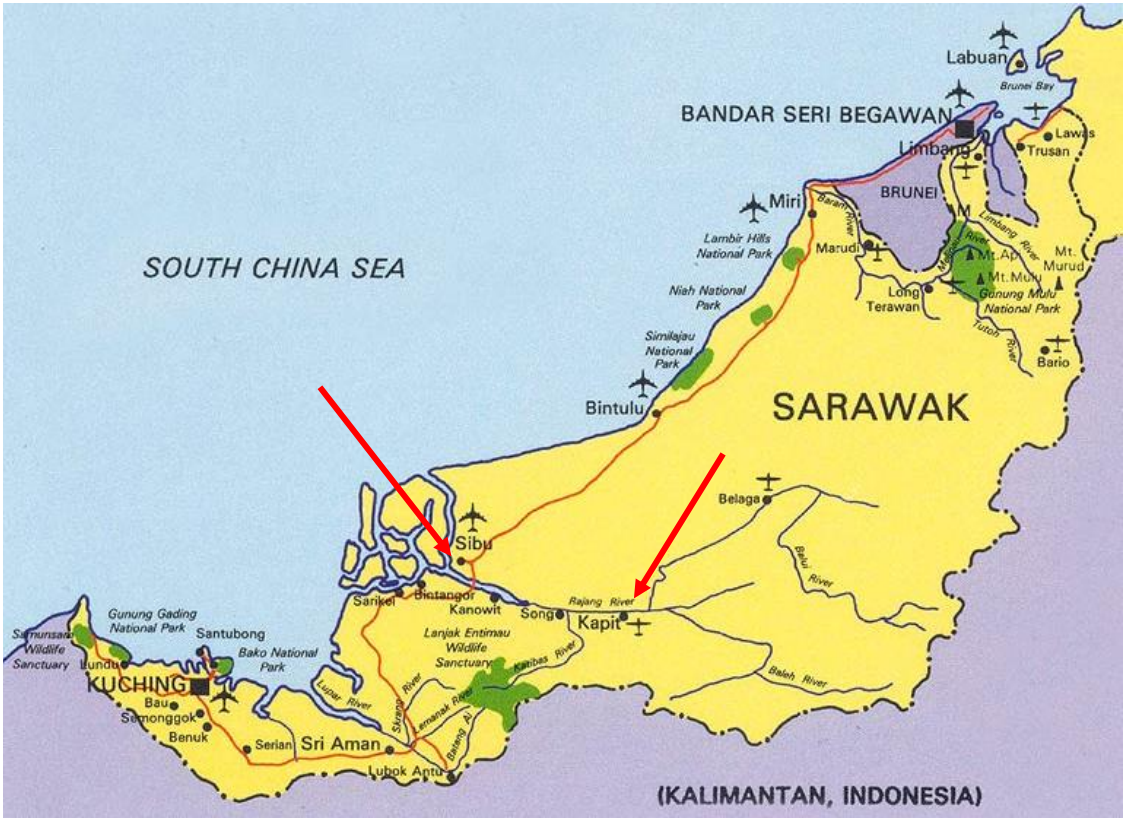

Figure 1: Map of Sarawak, Malaysia with arrows pointing to Sibu and Kapit. Source: "Map of Sarawak." *Tourist Attractions Malaysia*. http://www.touristattractionsmalaysia.com/sarawak/

diarrheal diseases, but such diseases continue to be a major cause of morbidity in the country.[2] Norovirus is the 8th leading cause of death due to diarrhea in children under five in Malaysia.[2] The highest cause of death due to diarrheal disease in Malaysian children under five years is *V. cholerae*, followed by *Salmonella* spp. and rotavirus. Although diarrheal disease represents a considerable disease burden in Sarawak, the disease-causing pathogens are not often identified.[20] Research has attempted to estimate the burden of rotavirus and *E. coli*, but estimates of the burden due to noroviruses is incomplete.[21,22] To address the substantial burden of diarrheal disease in Sarawak, Malaysia, it is important to identify the pathogens causing diarrheal disease, including norovirus.

1.4. Zoonotic Norovirus and One Health

Although norovirus has the potential to infect both humans and animals with species specific genotypes, little research has been done to identify norovirus zoonotic events, but existing research suggests animals may carry human norovirus. In 2008 in Japan, stool samples were collected from 240 healthy pigs and then tested for human and porcine norovirus genogroups. The researchers detected human norovirus genogroup II (GII) in 8 of the sampled pigs as well as porcine GII in 34 pigs, but not human genogroup I (GI).[15] Additionally, experimental evidence suggests that gnotobiotic pigs can be infected and show symptoms of disease from human norovirus GII.[23] In general, studies of swine stool samples for molecular evidence of human norovirus have yielded low prevalences, however serologic assessments of pigs have

found evidence of infection with human strains. In the United States, it has been estimated that 71% of pigs are seropositive for porcine norovirus and 63% and 52% of pigs are positive for human genogroups GI and GII, respectively.[24] This evidence suggests pigs are not necessarily presenting with norovirus disease symptoms, but they are developing an immune response to human viruses.

Little research has been carried out to understand the human risk for infection by porcine norovirus genogroups, but human and porcine genotypes of norovirus GII are very closely related.[25] This creates concern that species specific viruses could recombine to create viruses with unpredictable phenotypes and disease risks.[25] For example, porcine norovirus genotype QW101-like GII-18 is very closely related to human norovirus GII.[25] These two distinct viruses could recombine to create a new pathogenic virus.[25] Additionally, as pigs do not always show symptoms of norovirus infection, there is great potential that they could serve as reservoirs for emerging noroviruses.[25]

There is great concern that emerging and reemerging infectious diseases, such as norovirus, will put stress on public health systems. It is estimated that just over 12% of all human infections have recently emerged or are reemerging.[26,27] Animals are one source of these newly emerging and reemerging diseases, as roughly 58% of all human pathogens are zoonotic in origin.[26,27] Because the world is becoming increasingly connected with rapidly changing ecosystems, public health institutions need better solutions to solve the problem of emerging disease.[27] The One Health framework presents a potential solution in that it links stakeholders across disciplines to conduct

research to ultimately influence policy change.[27] According to the Centers for Disease Control and Prevention, One Health uses a "collaborative, multisectoral, and trans-disciplinary approach…with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment".[28] The term 'One Health' has also been adopted by the Food and Agriculture Organization, the World Health Organization, and the World Organisation for Animal Health.[29] One Health research has the potential to address the problem of emerging diseases because of the highly collaborative nature between human, animal, and environmental stakeholder groups.[30]

This study sought to estimate norovirus prevalence among children admitted to Sibu Hospital with diarrheal disease and in swine environments, and to understand the risk factors for novel zoonotic norovirus infections. The objective was to identify the prevalence of norovirus infections among individuals presenting with acute diarrhea at a large hospital in Sibu, Sarawak, Malaysia, and to identify zoonotic risk factors associated with these infections. Stool samples were collected from hospitalized patients at the time of enrollment. Additionally, in this study we sought to estimate the prevalence of norovirus among pigs in farms in Sarawak, Malaysia. Human and pig stool samples were analyzed for norovirus genogroups I and II. We hypothesized that, among patients with diarrhea, norovirus infections would be associated with a higher prevalence of previous exposure to domestic and wild animals.

2. Methods

This was a cross-sectional study seeking to estimate the prevalence of norovirus among children admitted to Sibu Hospital and in swine environments in central Sarawak, Malaysia. Stool samples were collected from children admitted to the hospital with acute gastroenteritis and from a number of area pig farms. Demographic information and animal exposure data were collected from children at the time of their enrollment.

2.1. Ethics

Field sample collection and laboratory procedures were carried out in biosafety level two (BSL2) conditions. Human participant enrollment and sample collection was approved by the Duke University Institutional Review Board and the Malaysian Medical Research and Ethics Committee. Swine sample collection was approved by the Institutional Animal Care and Use Committees at Duke University and the Animal Care and Use Committee at the Malaysian Ministry of Health.

2.2. Setting

Sarawak is a Malaysian state located on the island of Borneo with a population of 2.47 million people with more than 299,000 people living in Sibu, as of 2010.[18,31] Participants in the study were enrolled at Sibu Hospital. Sibu Hospital is the second largest hospital in the state of Sarawak with 635 beds and serves as the main referral hospital for Central Sarawak.[19] Patients admitted to the hospital with acute diarrhea in the pediatric ward were targeted for enrollment in the study.

In 2013, there were an estimated 542 pig farms with 1.38 million pigs throughout Malaysia.[32] There are 12 registered pig farms in Sibu, of which 11 were visited for sample collection. Visited farms ranged in size from small operations with less than 100 pigs to large farms with up to 7000 pigs.

2.3. Participant Enrollment and Sample Collection

At the time of admission, patients were screened for inclusion and exclusion criteria by medical officers in the pediatric ward. Inclusion criteria were being diagnosed with acute diarrhea at the time of enrollment and age greater than one month of age. Exclusion criteria were a confirmed laboratory diagnosis for a pathogen causing acute gastroenteritis other than norovirus or comorbid conditions requiring the patient to take laxatives. Medical officers then briefed the patients and/or their guardians about the study in the patient's primary language. If the patient and their guardian agreed to participate, they were given consent and enrollment forms in either Mandarin, Malay, or English. If the patient was a minor 7 to 18 years old, an assent form was also completed by the patient. After obtaining informed consent, medical officers then administered the survey to the participant or his/her guardian. The survey contained questions asking for demographic information as well as previous animal exposure and medical history. Enrollment information and survey responses were input into an online database using REDCap software (Vanderbilt University, Nashville, TN).

At the time of enrollment, a stool sample was obtained using FecalSwab collection kits (Copan Diagnostics, Inc., USA.). Stool samples were collected from fresh

diapers or from a bedpan. Stool samples were preserved in a stool collection cup at 4°C for up to 24 hours before being moved to a -80°C freezer for long-term storage and sample processing. Human stool samples were later shipped to Duke University in Durham, North Carolina for assay validation and further molecular testing. Each participant's questionnaire and stool sample was assigned a unique study identification number and was deidentified.

Swine stool samples were collected from registered farms in Sibu, Sarawak, Malaysia. At the time of sample collection, medical officers from Sibu Hospital interviewed farm owners or managers to obtain background information on the farm. Five fresh stool samples were collected by convenience sampling from pig pens at each farm using FecalSwab collection kits (Copan Diagnostics, Inc., USA). No more than one sample was collected per pig pen. At the time of collection, information about the age and number of pigs in the pen from which the sample was collected was recorded. Information about other animals present in the area of collection was also recorded. This was linked to the sample with a unique study ID. Stool samples were stored in a cooler with ice packs until they were moved to a -80°C freezer for long-term storage.

2.4. Molecular Analysis Molecular Analysis

Viral RNA was extracted from 200uL of pig stool samples using the spin protocol of the QIAamp cador Pathogen Mini Kit at the Clinical Research Center Laboratory at Sibu Hospital (QIAGEN, USA). After extraction, these samples were diluted ten-fold in Ultra-Pure water to prevent inhibition during molecular analysis and stored at -80°C.

Swine stool samples were tested for norovirus types I, II, and IV using previously described methods.[33,34] Viral RNA was amplified in swine stool samples using a one-step real-time reverse transcriptase protocol with SuperScript III/Platinum *Taq* DNA Polymerase (Thermo Fisher Scientific, Inc., Waltham, MA). Norovirus GI was amplified using forward primer NoV-for1B and probe NoV-probe1B added to a final concentration of 0.2uM and reverse primer NoV-rev added to a final concentration of 0.4uM with 5uL of extracted RNA. Norovirus GII and GIV were amplified using forward primers NoV-for2.1, NoV-for2.2B, NoVfor4 and probe NoV-probe2 added to a final concentration of 0.2uM and reverse primer NoV-rev added to a final concentration of 0.4uM with 5uL of extracted RNA. Viral RNA was amplified with initial reverse transcription and denaturation steps of 50°C for 15 minutes and 95°C for 2 minutes, respectively. This was followed by 40 cycles of denaturation and annealing and extension at 95°C for 15 seconds and 60°C for 30 seconds, respectively. PCR product was

Table 1: Primer and Probe Sequences for Molecular Testing

Name	Sequence	Target
NoV-for1B NoV-probe1B	TGGCAGGCCATGTTCCGCTGGATG **FAM –** TCGGGCAGGAGATTGCGATCTCCTGTCCA - **TAMRA**	GI*
NoV-for2.1 NoV-for2.2B NoV-for4 NoV-probe2	CAAGAGCCAATGTTCAGGTGGATG CAAGAGGCCATGTTTAGGTGGATG GAGTCCATGTACAAGTGGATG **FAM** – CTGGGAGCCAGATTGCGATCGCCCTCCCA – **TAMRA**	GII, IV*
NoV-rev	GCGTCATTAGACGCCATCTTCATT	GI, II, IV*
COG1F COG1R RING1	CGYTGGATGCGNTTYCATGA CTTAGACGCATCA TCATTYAC **FAM** – AGCACGTGGGAGGGCGATCG – **TAMRA**	GI†

*As described by Henke-Gendo, et al., 2009; Glowacka, et al., 2016.

†As described by Loisy, et al., 2005.

detected and analyzed using a CFX96 Real-Time PCR machine (Bio-Rad Laboratories, Inc., Hercules, CA).

Human stool samples were shipped to Duke University in Durham, North Carolina for molecular analysis. Viral RNA was extracted from human stool samples using the spin protocol of the QIAamp Viral RNA Mini Kit (QIAGEN, USA) at the Duke One Health Research Lab. A ten-fold dilution of sample in Ultra-Pure water was used prior to extraction to prevent inhibition during molecular analysis. Human stool samples were analyzed for norovirus types I, II, and IV with real-time reverse transcriptase PCR at the Duke One Health Research Lab (OHRL) in Durham, North Carolina.[33-35] Norovirus type II/IV viral RNA was amplified using the previously discussed methods and PCR product was detected using either a CFX96 Real-Time PCR machine (Bio-Rad Laboratories, Inc., Hercules, CA) or Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument (Applied Biosystems, Inc., Foster City, CA). Norovirus type I was amplified using the RNA UltraSense One-Step Quantitative RT-PCR System (Thermo Fisher Scientific, Inc., Waltham, MA). Forward primer COG1F, reverse primer COG1R, and probe RING1 were all added to a final concentration of 0.2uM with 5uL of extracted RNA.

For all reactions, ATCC quantitative synthetic RNA positive controls for norovirus genogroups I and II were used (ATCC, Manassas, VA). Ct values were recorded for each sample. A Ct value less than 38 was considered positive, greater than

or equal to 38 and less than 40 was a suspect positive, and any detection equal to or greater than 40 was negative.

2.5. Statistical Analysis

Statistical analyses were carried out using Microsoft Excel and STATA version 14.0 (Microsoft Corp., Redmond, WA; StataCorp LLC, College Station, TX). Age of participants in months was calculated in Microsoft Excel, and then categorical age variables were constructed in STATA. Age categories were 6 months old and younger, older than 6 months and 24 months old or younger, 24 months old to 60 months old, and older than 60 months. Descriptive statistics were generated for the demographic variables for ethnicity, household size, gender, and age in months. Frequency of exposures to pigs, cows, rodents, ducks, goats, chickens, dogs, cats, and other animals were calculated. Exposure was classified by whether or not the participant handled, cared for, or played with animals in the past two days.

Descriptive statistics were carried out on farm site data. Farm site data include farm size and average number of pigs present on a given day. Biosecurity information included other animals present at the farm.

Animal exposure frequencies were stratified by previous hospitalization due to acute diarrhea. Logistic regression was performed to obtain unadjusted odds ratios for exposure variables with the primary outcome of previous hospitalization from diarrheal disease because it is assumed that animal exposure remains constant over time. The outcome variable was previous hospitalization for diarrhea, and predictor variables

were general animal exposure, playing with animals, handling animals, caring for animals, cat exposure, dog exposure, and age category.

3. Results

3.1. Participant Enrollment and Farm Information

A total of 70 children were enrolled in the study from June 13 to August 1, 2017, ranging in age from 1 month to 10 years old. The average age was 30.8 months old. Of the participants enrolled, 43 were male (Table 2). A majority of patients enrolled were Iban, followed by Chinese, Melanau, Malay, and Bidayuh (Table 2). Of participants who identified as being of 'other' ethnicity, two were Kadazan, two were Kenyah, one was Dysun, and one was Punan. Households ranged in size from 3 people to 12 people. The average household size was 6 people, with the most common response being 5 members, which was 25.71% of the households (Table 2).

Information about animal exposure and the type over the course of norovirus incubation period of 48 hours was collected for all individuals enrolled in the study (Table 2). None of the individuals had any exposure to pigs, cows, rodents, ducks, goats, and chickens. Participants most commonly played with animals during the 48 hours prior to hospital admission, with 14.29% of children reported having played with cats, 5.71% of children reported playing with dogs, and 1.43% of participants played with rabbits (Figure 2). In the 48 hours before enrollment, 1.43% of children cared for and handled dogs (Figure 2). 5.71% of children handled cats 48 hours before being enrolled in the study, and 1.43% of children handled a rabbit (Figure 2). The most common animal children had recent exposure to was cats, with ten children having some type of cat exposure (Table 2). Four children had some level of dog exposure (Table 2).

Table 2: Demographic information and exposure frequency of individuals enrolled in the study.

Variables	N_{tot} = 70 (%)
Age	
1-12 months	24 (35.82)
12-24 months	17 (25.37)
2-5 years	13 (19.40)
5 years & older	13 (19.40)
Average age (months)	30.791
Ethnicity	
Iban	43 (62.32)
Chinese	11 (15.94)
Melanu	4 (5.80)
Malay	4 (5.80)
Bidayuh	1 (1.45)
Other	6 (8.70)
Sex	
Male	43 (61.43)
Female	27 (38.57)
Household size	
3	6 (8.57)
4	11 (15.71)
5	18 (25.71)
6	11 (15.71)
7	6 (8.57)
8	6 (8.57)
9	7 (10.00)
10	4 (5.71)
12	1 (1.43)
Previous hospitalization	
Yes	13 (18.57)
No	57 (81.43)
Norovirus II/IV infection	
Yes	3 (4.29)
No	67 (95.71)
Norovirus I infection	
Yes	-
No	-
Animal exposure	
Yes	13 (18.57)
No	57 (81.43)
Handled animals	
Yes	6 (8.57)
No	64 (91.43)
Played with animals	
Yes	13 (18.57)
No	57 (81.43)
Cared for animals	
Yes	1 (1.43)
No	69 (98.57)
Cat exposure	
Yes	10 (14.29)
No	60 (85.71)
Dog exposure	
Yes	4 (5.71)
No	66 (94.29)

Missing data for age (n=3) and ethnicity (n=1) variables

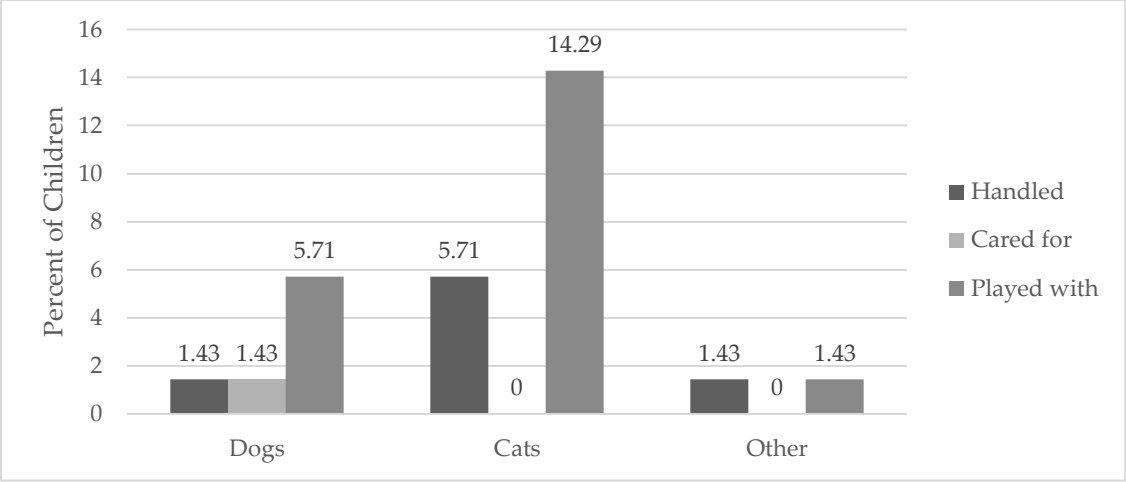

Figure 2: Self-reported animal exposure of individuals enrolled in the study during the 48 hours prior to enrollment.

A total of 11 farms were visited for sampling from June 30 to July 26, 2017, and at each farm five environmental pig stool samples were collected by convenience sampling. Farm size ranged from 0.0012 to 0.0243 km^2, with smaller farms having as few as 100 pigs on site each day and larger farms having up to 7000 pigs on site (Table 3). All farms visited had other animals on site. The most common animal in addition to pigs were dogs, with 49 samples collected in the vicinity of dogs (Table 3). Birds were also common near collection sites, with 19 samples near chickens, nine collected in areas with

Table 3: Descriptive information on farms and samples collected

Variables	
Farm and sample information	A
Farm size km^2 (avg.)	0.0012-0.243 (0.012)
Number of swine (avg.)	100-7000 (1103)
Animal age (weeks)	3 to 16
Samples taken from unhealthy pigs (%)	3 (5.45)
Other animals in the area	**N_{tot} = 55**
Dogs	49
Chickens	19
Ducks	9
Cats	9
Geese	5
Poultry	4
Rats	5
Geckos	5

ducks, five collected close to geese, and four close to other assorted poultry (Table 3). Rats and geckos were near five samples collected (Table 3).

3.2. Molecular Analysis

Molecular analyses on human stool samples were carried out at the Duke One Health Research Lab in Durham, North Carolina. After amplification by real-time reverse-transcriptase PCR, three samples were positive for norovirus genogroups II/IV. Molecular analyses of environmental pig stool samples were completed at the Sibu Hospital Clinical Research Center Laboratory in Sibu, Malaysia. No samples were positive for norovirus genogroups II/IV. Viral RNA amplification for norovirus genogroup I was unsuccessful in both human stool and pig environmental stool samples, as the assay could not be validated using the ATCC quantitative synthetic RNA positive control.

3.3. Patients Positive for Norovirus

Three individuals enrolled in the study had positive molecular test results for norovirus genogroups II/IV(NoV-GII/IV), resulting in a prevalence of 4.29% (Table 4). The youngest of these patients was 9 months old, followed by an individual 21 months old, and the oldest is 9 years old. Two of the patients were male, and the remaining

Table 4: Demographic characteristics of patients with a positive molecular test for norovirus-GII/IV.

Patient	Age (months)	Gender	Ethnicity	Household Size
20	21	Male	Chinese	4
42	9	Female	Chinese	6
45	111	Male	Iban	5

patient was female. Two of the individuals positive for NoV-GII/IV were Chinese and one was Iban. None of the individuals positive for NoV-GII/IV had any animal exposure in the past 48 hours, and none had been previously hospitalized for acute diarrhea. These individuals came from households with four, five, and six members (Table 4).

3.4. Previous Hospitalization for Acute Diarrhea

Thirteen individuals enrolled in the study had been previously hospitalized for acute diarrhea. Logistic regression was performed to calculate the unadjusted odds ratio for animal exposure variables with the primary outcome of previous hospitalization for acute diarrhea (Table 5). None of the exposure variables were significantly associated with increased odds of previous hospitalization for diarrheal disease. General animal

Table 5: Unadjusted odds ratios for the relationship between animal exposure and previous hospitalization for acute diarrhea.

	Previous Hospitalization		No hospitalization		
Variables	**N = 13**	**%**	**N = 57**	**%**	**Unadj. OR (95% CI)**
Animal exposure					
No	9	15.79	48	84.21	Ref.
Yes	4	30.77	9	69.23	2.37 (0.60, 9.39)
Handled animals					
No	12	18.75	52	81.25	Ref.
Yes	1	16.67	5	83.33	0.87 (0.09, 8.12)
Played with animals					
No	9	15.79	48	84.21	Ref.
Yes	4	30.77	9	69.23	2.37 (0.60, 9.39)
Cared for animals					
No	13	18.84	56	81.16	Ref.
Yes	0	0	1	100	-
Cat exposure					
No	9	15.00	51	85.00	Ref.
Yes	4	40.00	6	60.00	3.78 (0.89, 16.11)
Dog exposure					
No	12	18.18	54	81.82	Ref.
Yes	1	25.00	3	75.00	1.50 (0.14, 15.70)
Categorical age					
1-12 mos.	3	4.29	21	30.00	Ref.
12-24 mos.	1	1.43	16	22.86	0.44 (0.04, 4.61)
2-5 years	5	7.14	8	11.43	4.38 (0.84, 22.71)
>= 5 years	4	5.71	9	12.86	3.11 (0.58, 16.83)

exposure and playing with animals in the past 48 hours non-significantly associated with an unadjusted odds ratio of 2.37 (95% CI 0.60-9.39) (Table 5). Previously handling animals resulted in a non-significant decreased unadjusted odds ratio of 0.87 (95% CI 0.09, 8.12) (Table 5). An odds ratio for the exposure 'caring for animals' could not be obtained because the primary outcome of previous hospitalization for acute diarrhea was not present in all the cells. Prior cat exposure was trending on significance with an unadjusted odds ratio of 3.78 (95% CI 0.89-16.11), while dog exposure is not with an unadjusted odds ratio of 1.50 (95% CI 0.14, 15.70) (Table 5). None of the age categories led to significantly different odds ratios for previous hospitalization (Table 5).

4. Discussion

In this study, we enrolled 70 children admitted to Sibu Hospital for acute gastroenteritis to estimate the prevalence of norovirus and understand risks of animal exposure for developing diarrhea. Of the children enrolled, three tested positive for norovirus genogroup II (GII), while molecular assays for genogroup I (GI) were unsuccessful. Children were most frequently recently exposed to cats, with 10 children reporting cat exposure in the 48 hours prior to admission to the hospital. The most common type of animal exposure is having recently played with animals. Thirteen of the children enrolled in the study had previously been hospitalized for diarrheal diseases. Although none of the animal exposure variables significantly increase the odds of previous hospitalization for diarrhea, recent exposure to cats and playing with animals insignificantly respectively increased the odds by 3.78 (95%CI 0.89, 16.11) and 2.37 (95% CI 0.60, 9.39). Of the 11 farms visited, norovirus GII was not detected in any of the 55 pig environmental stool samples, norovirus GI assays were again unsuccessful. All samples were collected in the presence of other animals, with 49 collected near dogs and 19 collected near chickens.

4.1. Animals on Farms

All samples were collected in the vicinity of other animals, with dogs being most frequently in the area. This creates concerns about the possibility of noroviruses moving between species and recombining to create novel viruses. In other farms, both swine and human norovirus GII have been detected in fresh pen manure and pit manure.[36]

Additionally, human norovirus has been detected directly and through serology in dogs.[37,38] It is possible that pigs and dogs are serving as reservoirs of human norovirus, and their close proximity on farms in Malaysia increases the possibility of coinfection that could lead to viral recombination creating a novel virus.[36]

4.2. Animal Exposure

Because we did not have a large norovirus positive population in our study group, we decided to look at associations between animal exposure and previous hospitalization for acute diarrhea. It was assumed that animal exposure does not change over time, and, therefore, this outcome can serve as an interesting stand-in for norovirus infections. While the results of this study could not conclusively link prior animal exposure with norovirus and previous hospitalizations for diarrheal disease, other published evidence strongly suggests that exposure to animals, particularly feces, can cause diarrheal disease. A systematic review showed a significant positive association between domestic animal exposure and diarrhea, with the strongest associations occurring when the specific pathogen was identified.[39] Furthermore, this systematic review showed it was common for enteric pathogens to be transmitted between animals and humans.[39] A more recent systematic review published by the same group showed an additional association between diarrheal disease and exposure to domestic poultry and livestock in low and middle income countries.[40] These two systematic reviews suggest that there might be an association present between animal exposure and diarrhea in Malaysia, but it is likely that our sample size was too small to detect such an

association unless the true association had a very large true effect size. Additionally, because the association between human diarrheal disease and animals is stronger when the pathogen is identified, the limited positive patients for norovirus hindered our ability to quantify the association.

The strongest association we were able to show between animal exposure and diarrhea was the relationship between exposure to cats and previous hospitalizations for diarrhea. There has been a recognition for many years that dogs and cats can transmit diarrheal diseases to humans, as a review published in 1997 stresses this possibility.[41] More recently, a study showed that 13.1% of cats enrolled were infected with a zoonotic enteric pathogen.[42] Additionally, a case-report of a household showed the family's recent *C. jejuni* gastroenteritis was the fault of one of their newly adopted puppies.[43] While there is no significant association between cat exposure and hospitalization for diarrhea in this study, other research suggests a larger sample size would have added further support to the link between cat exposure and diarrheal disease. It is likely that playing with, handling, or caring for cats leads to an increased risk in children in Sarawak, Malaysia, and we suspect that a larger sample size would have added further support to the relationship.

An additional explanation for the lack of association between animal exposure and hospitalization for diarrhea lies in personal hygiene behavior of the participants. Personal hygiene behaviors highly affect one's risk for diarrheal disease following animal exposure. For example, in Vietnam, studies are unable to show an association

between animal contact and diarrhea, and they are also unable to show an association between owning livestock and morbidity or hospitalization from diarrhea.[39,44-46] It is possible that the association between diarrheal disease and animal exposure was not found in Vietnam because individuals there more commonly practice good personal hygiene and have higher overall nutrition.[44] Washing hands after contact with animals results in a decreased risk of moderate to severe diarrhea in children.[47] Although data on personal hygiene practices of participants were not collected in our study, other research suggests that Malaysians are aware of the importance of washing hands when handling food and in clinical settings.[48,49] Therefore, a relationship between diarrheal disease and animal exposure might not exist in Malaysia because their personal hygiene behaviors reduce the risk of disease.

4.3. Norovirus GI Assay

Despite closely following published protocols, all efforts to successfully validate the norovirus GI molecular assay were unsuccessful. However, other groups have also reported difficulties in detecting norovirus GI. A study carried out in 12 Canadian labs comparing the detection efficacy of different RT-qPCR protocols showed greater consistency between labs when detecting norovirus GII compared to GI.[50] Additionally, other newly developed detection methods show differences in sensitivity between GI and GII. Both immunochromatographic tests and nucleic acid sequence based amplification (NASBA) assays reported lower sensitivity for genogroup I than genogroup II.[51-53] Furthermore, when NASBA assays detected GI, the signal is often

much weaker than the signal for GII.[53] While the gold standard for detecting norovirus is RT-qPCR because of its published consistency at detecting various genogroups, the vast hereogeneity in the norovirus genome makes assay optimization difficult.[5,51,54,55] In this study, attempts to successfully validate the genogroup I assay were made over the course of months on site in the Sibu Clinical Research Center Laboratory and the Duke One Health Research Lab. Cycling conditions were altered and multiple enzymes were used to optimize the reaction. Unfortunately, we were unable to obtain a signal with the ATCC quantitative synthetic RNA positive control and therefore could not be sure that any results from the samples were accurate. However, unsuccessful validation of the genogroup I assays used in this study align with previous literature indicating assays targeting genogroup I have lower rates of detection compared to genogroup II.

4.4. Implications for Policy and Practice

Of the study group, only three patients tested positive for a norovirus infection. Therefore, norovirus represents a small proportion of the overall diarrheal disease burden in Sibu with a prevalence of 4.29%. Because of this, there is not sufficient need for Sibu Hospital to adapt norovirus diagnostics. However, because diarrheal diseases are still a significant burden in the hospital, Sibu Hospital should seek out other viral diagnostics to help understand the pathogenic burden in their patient population. Other viruses that are known to cause gastroenteritis are rotaviruses, astroviruses, enteric adenoviruses, and coronaviruses.[56] Adapting currently existing qPCR protocols to detect these viruses in patients with diarrhea in Sarawak, Malaysia will give doctors at Sibu

Hospital a better understanding of the disease burden in their patients. Additionally, because the risk factors for diarrheal disease are strongest when the pathogen of interest is identified, providing a diagnosis is important to understand what is putting children in Malaysia at risk for diarrheal disease.[39] Ultimately, understanding the risks for diarrheal disease can influence the design and implementation of programs reducing exposure to risks of interest to lessen overall diarrheal disease burden.

4.5. Implications for Further Research

Moving forward, future studies should include larger patient populations and include patients at different hospitals to understand the burden of norovirus and diarrheal disease in Sarawak, Malaysia. Additionally, future studies should search to establish the burden of diarrheal pathogens beyond norovirus. This expanded panel should include the top causes of pediatric diarrheal disease throughout the world, rotavirus, *Cryptosporidium* spp., and *Shigella* spp., as well as the top causes of pediatric diarrheal disease in Malaysia *V. cholerae* and *Salmonella* spp.[2] Additionally, other known diarrheal viruses should be included in this pane, such as rotaviruses, astroviruses, enteric adenoviruses, and coronaviruses.[56,57] These pathogens will be detected with qPCR, eliminating the need for cell or bacterial culture in the research lab.

In order to understand more of the causes of diarrhea in Sarawak, Malaysia, future studies should include information inquiring about the personal hygiene and water and sanitation access of patients. Because handwashing and access to clean water and improved sanitation decreases risk of diarrheal disease, it is necessary to

understand these behaviors and environmental factors in the patient population.[3,47,58-60] Additionally, future studies should also attempt to more clearly define the nature of zoonotic noroviruses in Malaysia. Studies suggest that pet dogs are able to transmit norovirus to their owners, with an estimated 4.3% of dogs testing positive for human norovirus and almost universal seropositivity in dogs against human norovirus.[37,38] Although there was limited dog exposure in the study participants, previous research suggests pets can transmit diarrheal disease to the household. To capture these potential zoonotic events, individuals who are diagnosed with acute gastroenteritis should be queried about the presence of pets and other domestic animals in the house. If the household has pets, the family will be asked to provide a stool sample from the animals. The complete diarrheal disease panel will be run on both the sick individual's and the pet's stool sample to determine if zoonotic transmission occurred.

Due to the difficulties with implementing qPCR in resource limited settings, it is incredibly important that novel methods be developed to detect pathogens. One promising method of detecting pathogens in the field is serology saliva testing. Salivary tests have been shown to successfully detect an immune response to a variety of environmental pathogens, including norovirus.[61] Additionally, salivary serologic tests have an added benefit in that they do not require as much labor or time compared to traditional molecular tests.[62] These tests can provide a stronger estimate of past burden of disease because they do not rely on self-reporting of disease symptoms and can determine previous exposures.[63] However, many of these salivary serologic tests are still

being validated. Therefore, bringing them to Malaysia to test for diarrheal disease would help validate their use while also establishing the prevalence of pathogens in settings where less is known.

4.6. Study Strengths and Limitations

There are a few limitations to the study. First is the sample size; because there were only 70 patients, our study did not have enough statistical power to detect any significant associations between animal exposure variables and the primary outcome of norovirus. Additionally, this study was limited by the duration of the study. Results are not generalizable beyond the summer months because norovirus is a seasonal virus. This study was also strongly limited by the success of the molecular assays. Because the molecular assay detecting norovirus GI could not be validated, we were unable to test samples for GI. However, this study has strengths in that it is the first of its kind in Sarawak, Malaysia. Results of the study were able to inform medical providers at Sibu Hospital of the burden of norovirus. A further strength of this study is its multi-faceted approach to understand zoonotic norovirus in Sarawak, Malaysia because it samples from both humans and pigs.

5. Conclusion

This study was the first to attempt to understand the burden of norovirus and animal exposure risk factors for diarrheal disease among children with acute diarrhea in Sibu Hospital in Malaysia. Additionally, no previous studies have tried to detect zoonotic norovirus in swine environments in Malaysia. Although we were unable to identify any statistically significant risk factors for diarrhea, results suggest that cat exposure puts children at increased odds of being hospitalized for diarrhea. This indicates that domestic animal exposure might be a risk factor for severe diarrheal disease in Malaysia.

In order to reduce the burden of diarrheal disease in Sarawak, Malaysia, the pathogens causing disease and the source of those pathogens must be elucidated. To accomplish this, better diagnostic methods for diarrheal pathogens must be developed. One promising method for detecting viral pathogens in resource limited settings is salivary serologic tests. These have the benefit of understanding the current disease-causing pathogen in addition to past exposure. The results of the current study indicate that noroviruses are not highly prevalent in children in Malaysia. However, the overall burden of diarrheal disease at Sibu Hospital suggests that the Clinical Research Center would benefit from expanding their diarrheal diagnostics to better treat and understand diarrheal disease in their patients.

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