UNIVERSITI PUTRA MALAYSIA

MOLECULAR DETECTION OF NOROVIRUS IN PORCINE, CANINE AND FELINE IN SELANGOR, MALAYSIA

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MOLECULAR DETECTION OF NOROVIRUS

IN PORCINE, CANINE AND FELINE IN SELANGOR, MALAYSIA

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FACULTY OF VETERINARY MEDICINE

UNIVERSITI PUTRA MALAYSIA

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MOLECULAR DETECTION OF NOROVIRUS

IN PORCINE, CANINE AND FELINE IN SELANGOR, MALAYSIA

TAN WEI YANG

A project paper submitted to the

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Molecular detection of norovirus in porcine, canine and feline in Selangor, Malaysia” by Tan Wei Yang and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD4999 – Final Year Project.

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DEDICATION

This thesis is especially dedicated to:

My loving parents

Tan Keyok Bong and Fung Fong Pin

My supportive supervisors

Prof. Dr Siti Suri Binti Arshad

Assoc. Prof. Dr Ooi Peck Toung

Assoc. Prof. Dr Gayathri Thevi Selvarajah

And

DVM2021 Batchmates

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TABLE OF CONTENTS

	Page
TITLE	**i**
CERTIFICATION	**ii**
DEDICATION	**iv**
ACKNOWLEDGEMENTS	**v**
TABLE OF CONTENTS	**vi**
LIST OF TABLES	**vii**
LIST OF FIGURES	**x**
LIST OF APPENDICES	**xi**
ABREVIATIONS	**xii**
ABSTRAK	**xiv**
ABSTRACT	**xvi**

CHAPTER 1.0: INTRODUCTION

1.1	Norovirus	1
1.2	Porcine norovirus	2
1.3	Canine norovirus	2
1.4	Feline norovirus	3
1.5	Objective and justification	4
1.6	Hypothesis	6

CHAPTER 2.0: LITERATURE REVIEW

2.1.1	Overview of Norovirus	7
2.1.2	Porcine norovirus	8
2.1.3	Canine norovirus	9
2.1.4	Feline norovirus	10
2.2	Virus structure and genome, molecular characteristics	11
2.3	Norovirus clinical manifestations and pathology	
2.3.1	Clinical manifestations and pathology in human	13

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2.3.2 Clinical manifestations and pathology in animal 14
2.4 Norovirus transmission 15
2.5 Norovirus detection method 19

CHAPTER 3.0: MATERIALS AND METHODS

3.0 IACUC approval 23
3.1 Animal selection 23
3.2 RNA extraction 25
3.3 Reverse transcription 26
3.4 Primer selection 28
3.5 Positive control selection 30
3.6 Polymerase chain reaction 31
3.7 Agarose gel electrophoresis and photography 34

CHAPTER 4.0: RESULTS

4.1 Sampling of animals 36
4.2 Measurement of RNA concentration and purity 36
4.3 Optimisation of universal primer detecting norovirus on positive controls Norovirus group I (Human NoVs) and Norovirus group II (synthetic and human NoVs) 36
4.4 Detection of Norovirus in multiple animal species by RT-PCR assay using Universal Primers 37
4.5 Detection of Norovirus in porcine, canine and feline faecal samples by using RT-PCR assay using species specific primers 41

CHAPTER 5.0 DISCUSSION

5.1 Detection of Norovirus in swine, canine and feline in Selangor via RT-PCR Assay 42

CHAPTER 6.0 CONCLUSIONS 47

CHAPTER 7.0 RECOMMENDATIONS 48

REFERENCES 49

APPENDICES 53

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LIST OF TABLES

		Page
Table 3.1:	Total number of porcine faecal sample obtained from Selangor state used for the detection of porcine norovirus	23
Table 3.2:	Total number of canine faecal sample obtained from an animal shelter in Selangor state used for the detection of canine norovirus	24
Table 3.3:	Total number of feline faecal sample obtained from an animal shelter in Selangor state used for the detection of canine norovirus	25
Table 3.4:	Genomic DNA removal reaction components	27
Table 3.5:	Reverse-transcription reaction components	28
Table 3.6:	Universal primers used for DNA amplification of norovirus	29
Table 3.7:	Porcine specific primers used for DNA amplification of porcine norovirus	29
Table 3.8:	Canine specific primers used for DNA amplification of canine norovirus	29
Table 3.9:	Feline specific primers used for DNA amplification of feline norovirus	30
Table 3.10:	Cycling conditions of conventional PCR assay for universal primer for detection of norovirus	32
Table 3.11:	Cycling conditions of conventional PCR assay for porcine specific primer for detection of porcine norovirus	32
Table 3.12:	Cycling conditions of conventional PCR assay for canine specific primer for detection of canine norovirus	33
Table 3.13:	Cycling conditions of conventional PCR assay for canine specific primer for detection of feline norovirus	33

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Table 4.1:	Prevalence rates of norovirus in porcine, canine and feline from farms and animal shelter in Selangor following PCR assay by using universal primer on faecal samples	40
Table 4.2:	Frequency data of positive Porcine Norovirus (NoVs) in the faecal sample with open house management and closed house management	41

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LIST OF FIGURES

		Page
Figure 4.1	PCR assay for detection of NoV RdRp gene in positive control samples was done by using universal primer pair p290/p289	37
Figure 4.2:	RT-PCR amplification on porcine samples using universal primer	38
Figure 4.3:	RT-PCR amplification on canine samples using universal primer	38
Figure 4.4:	RT-PCR amplification on feline samples using universal primer	39

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LIST OF APPENDICES

		Page
Appendix 1:	Pictures of sample collection, processing and results reviewing	53
Appendix 2:	Design of Norovirus gene construct.	54
Appendix 3:	IACUC approval letter	56
Appendix 4:	RNA concentration of each extracted faecal samples.	57

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ABBREVIATIONS

BSC	Body score condition
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
°C	Degree Celsius
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ELISA	Enzyme-linked Immunosorbent Assays
g	Gram
gDNA	Genomic deoxyribonucleic acid
IACUC	Institutional Animal Care and Use Committee
ID	Identity
kb	Kilobase
µg	Microgram
µl	Microliter
µm	Micromolar
min	Minutes
ml	Millilitre
mg	Milligram
NoVs	Norovirus
nt	Nucleotide
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction

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RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcription polymerase chain reaction
x *g*	Relative centrifugal force
USA	United States of America
V	Volt

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ABSTRAK

PENGESANAN MOLEKULAR NOROVIRUS PADA KHINZIR, ANJING DAN KUCING DI SELANGOR, MALAYSIA

Oleh

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Norovirus (NoVs) diklasifikasikan dalam keluarga. Caliciviridae, positif sense, RNA berbenang satu. Norovirus merupakan sebab utama yang mengakibatkan sakit perut pada manusia yang disebabkan oleh agen bukan bakteria di dunia. Walaupun norovirus dikesan di seluruh dunia, tetapi tiada data untuk norovirus pada haiwan seperti khinzir, anjing dan kucing. Oleh sebab itu, kajian ini dijalankan dengan tujuan untuk mengkaji norovirus pada haiwan sihat tempatan termasuk khinzir, anjing dan kucing. Dalam kajian ini, 24 khinzir termasuk 12 babi menyapih dan 12 babi bertina dewasa, 20 anjing dan 20 kucing telah dipilih untuk kajian ini. Tinja khinzir telah dikumpul dari 2 ladang khinzir di Selangor dan untuk anjing dan kucing, najis telah diambil di agensi perlidungan haiwan di Selangor. Tinja telah diproses mengikut teknik molekular esei RT-PCR. Projek ini telah mengunakan 4 primers yang telah diterbitkan. Untuk primer sejagat (1 set), ia mensasar kawasan RdRp manakala untuk primer spesies khusus (4

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set), ia mensasar kawasan kapsid. Keputusan yang diperolehi dalam kajian ini ialah 0% (0/46), 0% (0/20) dan 0% (0/20) untuk khinzir, anjing dan kucing adalah positif untuk antigen norovirus masing-masing. Walau bagaimanapun, keputusan ini tidak mengesahkan norovirus tidak wujud pada haiwan di Malaysia kerana virus ini terdapar pada negara jiran lain. Kegagalan pengesanan norovirus dalam sampel tinja berkemungkinan besar disebabkan oleh penyampilan haiwan sihat, di mana mengikut laporan, rembesan norovirus dalam tinja hanya dalam tempoh 5 hari dalam tempoh jangkitan akut. Oleh itu, kajian ini digalakkan untuk memperolehkan sampel daripada haiwan jangkitan aktif dengan masalah gastroenteritis. Di samping itu, kajian serologi boleh dilakukan untuk mengesan antibodi terhadap NoVs untuk mengesan haiwan yang mempunyai pendedahan awal terhadap NoVs. Kesimpulannya, NoVs antigen tidak dapat dikesan pada khinzir, anjing dan kucing yang sihat di Selangor.

Kata kunci: *norovirus, Malaysia, RT-PCR, pengesanan molecular*

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ABSTRACT

MOLECULAR DETECTION OF NOROVIRUS IN PORCINE, CANINE AND FELINE IN SELANGOR, MALAYSIA

By

Tan Wei Yang

Matric No.: 188722

Supervisor: Prof. Dr Siti Suri Arshad

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Norovirus (NoVs) belongs to the Caliciviridae family, non-enveloped, positive sense, and single-stranded RNA viruses. Norovirus is the leading cause of nonbacterial gastroenteritis in humans worldwide. Despite the world-wide norovirus detection, there is still no data on norovirus on porcine, canine and feline in Malaysia. Thus, this study aimed to describe norovirus locally in healthy animals which include porcine, canine and feline. In this study, 24 pigs including 12 weaners and 12 sows, 20 dogs and 20 cats faecal samples were selected for this study. The faecal samples of porcine were collected from 2 pig farms in Selangor, while faecal samples for canine and feline were collected from an animal shelter in Selangor. Faecal samples were processed according to standard virology and molecular methods and subjected to RT-PCR assay. There were 4 published primers used consisting of universal primers targeting the RdRp

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region (1 set) and species-specific primers targeting the capsid region (3 sets). Results revealed that 0% (0/46), 0% (0/20), and 0% (0/20) of the porcine, canine and feline were positive for NoVs antigen, respectively. This finding, however, does not rule out the absence of norovirus in animals, as the virus is prevalent in several countries. Unsuccessful detection of NoVs in faecal samples could be attributed mainly to sampling of healthy animals where according to report NoV shedding in faecal last for only 5 days during acute infection. Thus, it is highly recommended for the samples to be obtained from acutely infected animals with gastroenteritis signalment. Alternatively, serological study could be done by detecting antibodies against NoVs to detect animals that have past exposure to NoVs. In conclusion, NoVs antigen could not be demonstrated in clinically healthy porcine, canine and feline in Selangor.

Key words: *norovirus, Malaysia, RT-PCR, molecular detection*

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INTRODUCTION

1.1 Norovirus

Norovirus (NoVs) belongs to the Caliciviridae family, non-enveloped, positive sense viruses with diameters of 38 nm and consist of small, circular, single-stranded RNA genomes. Caliciviruses have a broad host range including humans, pigs, cattle, dogs, sheep and rabbits. The virus induces a wide spectrum of diseases and lesions in digestive tract infection, upper respiratory infection and stomatitis (Atmar *et al.*, 2008). Currently, norovirus has become the common cause of food- and waterborne, acute, viral gastroenteritis in the human population worldwide (Hutson *et al.*, 2004). In the United States, NoVs are accountable for over 90% of food-related outbreaks of gastroenteritis (Mead *et al., 1999*). For transmission, NoVs is transmitted through contaminated food consumption (particularly oysters) or water, by faecal–oral route, person-to-person, via airborne route or through contaminated surfaces contact. NoVs is able to infect humans of all ages throughout the whole year but the infections are mostly in winter months (Hutson *et al*., 2004).

Noroviruses are genetically diverse and currently classified into five genogroups according to the amino acid sequence alignment for the partial RNA-dependent. It is highly heterogeneous due to genetic recombination. There are many different types of noroviruses, so humans can be infected with norovirus illness many times in their life. Infection with one type of norovirus will not protect against other types of genotype but it is possible to gain immunity (protection against) to specific types. For the immunity,

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it is not known exactly how long it lasts. During norovirus outbreaks, many people of all ages get infected due to the nature of norovirus infection (Henry, 2019).

1.2 Porcine Norovirus

Animal NoVs can cause gastroenteritis in the swine population (Smith *et al.,*2012). Recently, public health concerns about zoonotic transmission of porcine NoVs to humans arose when Genogroup II norovirus (GII NoV) RNA was detected from swine. Caliciviruses were found in the faecal samples of pigs and calves using electron microscopy soon after the discovery of the Norwalk virus (Bridger *et al.,* 1980). In the 1990, the first porcine NoV was detected in a healthy pig and raised the attention to the zoonotic potential of caliciviruses (Summa *et al*., 2012). NoVs detected in naturally infected pigs are classified as GII, which can infect both porcine and humans. GII.11, GII.18 and GII.19 are recognized as porcine NoVs and the remaining 16 GII genotypes are human viruses (Wang *et al.,* 2005).

1.3 Canine Norovirus

The detection of a novel GIV and GVI NoVs norovirus in a young dog (Martella *et al.,* 2008) proved that dogs can be infected with NoVs. On the other hand, GIV NoV has also been detected in a cat and a lion (Martella *et al.,* 2008). In 2007, major human NoV (HuNoV) genogroup GII was also detected in the faecal of 3 dogs (Martella, 2008). In 1983, dogs were the first animal to be suggested as potential zoonotic vectors of HuNoV, following an outbreak of norovirus gastroenteritis in an old folk home just before the finding of norovirus isolated from stools of the owner (Henry, 2019). In one study, researchers found that the essential step for Human NoV infection of cells

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requires Human NoV to bind on the complex carbohydrates known as histo-blood group antigens (HBGAs) (Marionneau *et al.,* 2001). HBGAs are found on erythrocytes, on epithelial cells surface of the gastrointestinal, genitourinary, and respiratory tracts. This HBGAs can be secreted by these cells into body fluids like saliva (Marionneau *et al.,* 2001).

Following human NoV attachment to HBGAs in vitro, the internalization of viral particles into cells denotes that the primary step for HuNoV uptake into cells is the binding of HuNoV to the HBGAs receptor. This study further indicates that the susceptibility to Human NoV infection is related to the expression of HBGAs in the gastrointestinal tract (Hutson *et al.,* 2004). For dogs to be susceptible to HuNoV, the dogs must express HBGAs in their gastrointestinal tracts. With the increase of the household dog population in Malaysia, the possibility of HuNoV cross species between humans and animals has raised a public health concern.

1.4 Feline Norovirus

In cat, there is a well-known respiratory pathogen in cats, called feline calicivirus (FCV). In one study, researcher found that GIV NoV has been detected in a cat and a lion (Martella *et al.,* 2008). Several studies have documented that NoVs also circulate in carnivores. The first NoV in carnivores was detected in captive lion cubs with severe enteritis (Martella *et al.,* 2008). In cats, feline NoV (FNoV) was detected in domestic cats in 2012 (Pinto *et al.,* 2012). In that study, FNoV is found in 2–3-month-old cats that having gastroenteritis in an animal shelter. This finding suggestive of feline norovirus can lead to gastroenteritis problem. However, there is no report on the in vivo

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studies to elucidate that cat infected with FNoV lead to gastroenteritis. Based on the full-length VP1, the lion and dog NoVs were classified as a distinct genotype GIV.2 within GIV, along with human Alphatron-like NoVs (GIV.1) (Bank-wolf *et al.,* 2010).

1.5 Objective and Justification

The genetic similarities between human and porcine norovirus have raised public health concerns regarding the possibility of the emergence of new epidemic human strains (Mattison *et al.,* 2007). Since the norovirus genus groups are shared amongst animals and humans, where the virus genogroups could possibly infect human and animal hosts, there is a high chance of zoonotic transmission of infection between human and animal hosts (Mattison *et al.,* 2007).

Currently, the status of norovirus in animal hosts in Malaysia is still unknown. Norovirus is detected in many different regions of the world and it is found in porcine, canine, feline, ruminant and wildlife species (Martella *et al.,* 2008). The increased number of closed house management of porcine farms in Malaysia with air-conditioned facilities has tremendously improved the production by controlling several important pig diseases. The cool temperature in the farm setting and breeding the imported temperate pig breed such as from Denmark and Belgium could possibly serve as predisposing factors for norovirus infection. In addition to farm animals, the increased household pets also raised a concern of zoonotic and reverse zoonotic transmission of norovirus between owner and pets. NoV was reported in pigs in several neighboring countries including Thailand, Taiwan and Japan (Chao *et al.,* 2012). Although recent screening of pig samples for norovirus following human norovirus cases in Sarawak

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show seronegative (Philo *et al.,* 2018), the potential of NoV to spread between hosts cannot be ruled out. The geographical distribution of Norovirus reported in several countries is highly suggestive that NoV is ubiquitous and is likely to be detected in Malaysia. Thus, porcine, canine and feline are chosen for the preliminary study to determine the presence of Norovirus antigen in animal hosts. Since, there is no report on norovirus in multiple animal species in Malaysia, this study will be the first of its kind. Results obtained will provide a database on norovirus status in animals and serve as a steppingstone for further research of norovirus.

The study was conducted with the following objectives:

1. To detect the presence of norovirus in faecal samples of porcine by using RT-PCR method.

2. To detect the presence of norovirus in faecal samples of canine by using RT-PCR method.

3. To detect the presence of norovirus in faecal samples of feline by using RT-PCR method.

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1.6 Hypothesis

Null hypothesis 1: Norovirus is not detected in faecal samples of porcine.

Alternative hypothesis 1: Norovirus is detected in faecal samples of porcine.

Null hypothesis 2: Norovirus is not detected in faecal samples of canine.

Alternative hypothesis 2: Norovirus is detected in faecal samples of canine.

Null hypothesis 3: Norovirus is not detected in faecal samples of feline.

Alternative hypothesis 3: Norovirus is detected in faecal samples of feline.

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LITERATURE REVIEW

2.1.1 Overview of norovirus

Noroviruses (NoVs) belong to the Caliciviridae family, of Norovirus genus cause diarrhea in humans and animals (Zheng *et al.,* 2006). Norovirus (NoV) is the most common non-bacterial cause of foodborne gastroenteritis worldwide. in many countries including Malaysia (Villabruna *et al,* 2019). NoV is classified into genogroup I (GI) to genotype 5 (GV) by molecular characterization based on the partial or complete capsid or RNA-dependent RNA polymerase (RdRp) sequence. Additional genogroups GVI and GVII have recently been added (Martella *et al.,* 2008). GI, GII, GIV and GVI are known to infect humans and GII is the most prevalent (Zheng *et al*., 2006). For GI and GII groups, they are further classified into many genotypes and with the discovery of new strains, the classification is in constant change (Nakamura *et al*., 2010).

NoVs have been reported to be detected in several animal species including swine, cattle, mice, lions, dogs, bovine and murine. In carnivores, the strains of GIV and GVI NoVs have been detected in dogs (Mesquitta *et al*., 2010). For the strains in bovine and murine, there are classified as GIII and GV, respectively genetically different from human strains (Karst *et al.,* 2003).

In the United States, human NoVs can cause an estimated 23 million cases of illness annually in the United States (Mead *et al*., 1999) and more than 90% of nonbacterial epidemic gastroenteritis worldwide (Green *et al.,* 2001). There are several aspects that make human NoVs highly contagious which are low infectious dose, environmental resistance, strain diversity, shedding from asymptomatic persons, and

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varied transmission vehicles. For local cases, the first study was done on norovirus outbreak among students of a boarding school in Kluang, Johor, Malaysia. In that study, twelve fresh stool and 14 food samples were tested for NoV and eight (67%) stool samples and six (43.9%) food samples were positive for NoV and total respectively. This was the NoV outbreak that was determined for the first time in Malaysia. There was study in Malaysia during an outbreak of diarrhea in a kindergarten in Sabah, Malaysia. In that study, stool samples from seven children and 13 teachers were tested for rotavirus and norovirus. All samples were negative for rotavirus but positive for norovirus. To find out the genogroup and genotype of norovirus, nucleotide sequencing of the amplicons was performed. All norovirus from this outbreak was of genotype GII.2 (Martella *et al.,* 2008).

2.1.2 Porcine norovirus

Swine is susceptible to NoV GII and this genogroup is the most prevalent cause of acute viral gastroenteritis in humans. Porcine strains are associated with human strains genetically and antigenically (Farkas *et al*., 2005). The first porcine NoV was found in a healthy pig in 1990 (Sugieda *et al.,* 1998). Human NoV strains replicate and induce mild infection in gnotobiotic pigs (Cheetham *et al.,* 2006), which support zoonotic transmission of noroviruses (NoVs). Animal NoVs can cause gastroenteritis in several animal species include swine, calves, dogs and mink (Smith *et al.,* 1983). Three of the NoV genotypes namely the GII.11, GII.18 and GII.19, are recognized as porcine NoVs and the remaining 16 GII genotypes are human viruses. The genetic resemblances between human and porcine NoVs have raised public health, the possibility of zoonotic

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transmission between animal and human and the emergence of new epidemic human strains (Cheetham *et al.,* 2006). In Japan, Porcine NoVs were first reported in Shizuoka Prefecture in Japan in 1997 (Nakamura *et al.,* 2010) where the GII.11 genotype was detected in healthy pigs. Later porcine NoV is reported in many other countries such as the United States (Cheetham *et al.,* 2006), Belgium, China and Brazil (Silva *et al.,* 2015). In Japan, GII.11, GII.18, and/or GII.19 genotypes of PoNoVs VP1 genes have been detected in both apparently healthy and diarrheic pigs (Nakamura *et al*., 2010). In China, there is a recombinant PoNoV strain detected in a diarrheic pig and this recombinant strain induces diarrhoea to specific pathogen-free miniature pigs (Shen *et al.,* 2012). The tendency of NoV to mutate has raised a concern of emerging pandemic human strain. Human noroviruses (HuNoVs) have also been detected in pigs (Mattison *et al*., 2007 and Nakamura *et al.,* 2010), demonstrating that pigs are susceptible to HuNoVs infection. Although the NoVs are mutating, (Nakamura *et al*., 2010) the genetic variation of each genotype of porcine NoV was limited, that may indicate there is low chance for newly emerging recombinant porcine NoVs to occur. There is one study that found antibodies detected against human NoVs among pigs in Venezuela.

2.1.3 Canine norovirus

Canine noroviruses were first reported in 2007 in Italy and the strain was group as GIV.2 genotype (Martella *et al.,* 2008). These viruses have been found to cause diseases in dogs in Asia and Europe following the detection of canine NoVs in stool samples from dogs in Portugal (Mesquita *et al.,* 2013). Subsequently, the seropositivity for canine NoV was estimated to be 39% in dogs from 14 different European countries

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(Mesquita *et al.,* 2013). In the United Kingdom, the seroprevalence of human noroviruses in dogs was reported to be 13% (Caddy *et al.,* 2013). In Finland, GII.4 genotype of human NoV origin (variants GII.4-2006b and GII.4-2008) was detected in their local dogs, and this finding may prove that human noroviruses can be transmitted to canine and cause gastroenteritis in dogs (Summa *et al.,* 2012). In humans, antibodies against canine norovirus were also reported in veterinarians with high risk of exposure to canine (Mesquita *et al.,* 2013). The emergence of Canine NoVs has become a public health concern as pets are an integral part of family life in most countries including Malaysia. Their close relationship with humans needs to have special attention with potential reservoirs of zoonotic agents (Summa *et al.,* 2012).

2.1.4 Feline norovirus

Feline norovirus of genogroup IV (GIV.2) was detected in cats and lions (Otto *et al.,* 2011). Feline NoVs were detected in the feces of a captive lion cub with severe hemorrhagic enteritis in Italy (Mochizuki, 2002) and cats with a history of vomit and diarrhea in Germany (Humphery *et al.,* 1984). In Italy and Greece, NoVs genetically related to the lion NoV were detected in the fecal samples of dogs with diarrhea. Based on the full-length VP1 gene, the lion was classified as a distinct genotype GIV.2 within genotype IV (GIV), along with human Alphatron-like NoVs (GIV.1) (Bank-wolf *et al.*, 2010).

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2.2 Virus structure and genome, molecular characteristics

Noroviruses belong to the Caliciviridae family. Caliciviridae is named after its cup-like pockets morphology on their surface Norovirus, has RNA polymerase (RdRp) and major capsid protein (Nakamura *et al,* 2010). The virus capsid causes infection and protects viral RNA and viral genome (Martellla *et al.,* 2017). The virus capsid is composed of 180 molecules of a single protein and each molecule is called a monomer where each of the monomers self-assemble into dimers. These 90 dimers in turn accumulate into the virus capsid that is made of two basic structural components which are the shell and the linked flexible hinge that protrudes. The shell is known as "S", is the structural core of the virus that surrounds the genome. It comprises the combined shell domains of all the monomers. The protruding region, known as "P", consists of the combined protruding domains of all the monomers. The P domain is subdivided into two subdomains, P1 and P2, with P2 being the outermost subdomain.

Noroviruses are genetically diverse. The virus comprises up to 27 genotypes within 7 genogroups, based on the capsid genes of 164 strains (Zheng *et al.,* 2006; Martella *et al.,* 2008). 31 genetic clusters (genotypes) have been found within these genogroups, including 1–8 within genogroup I (GI), 1–19 within GII, and only one genotype within each GIII, GIV and GV (Zheng *et al.,* 2006). Human gastroenteritis has been associated with NoVs GI, GII and GIV (Nakamura *et al.,* 2010). In another study, it was found that GI, GII, GIV and GV are known to infect humans; where GII being the most prevalent genogroup (Zheng *et al.,* 2006). GI and GII are further divided into

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many genotypes and with the discovery of new strains the classification is in constant evolution (Nakamura *et al.,* 2010).

The viral genome ranges from 7.4 to 7.7 kb in length and is organized into three or four open reading frames (ORFs). The 5′ proximal ORF1 encodes a non-structural polyprotein which is cleaved into six mature products by the virally encoded protease (NS6, or Pro). The protein can be divided into shell S and protruding (P) domains, and the P domain further subdivided into the P1 stalk domain and the hypervariable P2 domain containing the tips of the arches. Besides, ORF3 encodes the minor structural protein VP2 and ORF4. Murine NoV genomes encode a newly defined protein called virulence factor 1, or VF1. NoV genomes are covalently linked to VPg at their 5′ ends and polyadenylated at their 3′ ends. For ORF3, it encodes a minor structural protein called VP2. VP1 and VP2 are translated from a sub genomic RNA. For murine NoVs, a fourth of overlaps ORF2 lead translation from this alternative ORF4 produces a recently identified protein virulence factor 1 (VF1) (Thackray *et al.,* 2007).

The NoV RdRp is a good target for drug advance based on its commonalities to other more widely studied viral RdRp enzymes for which many antiviral drugs have been developed. The NoVs replication complex is related with virus-induced intracellular membranous vesicles (Wang *et al.*, 2005). Duplication complex formation also consists of the cytoskeletal network, allowing the formation of the replication complex in proximity to the microtubule organizing network (Wang *et al.*, 2005).

The human NoV replicon system, by substituting the ORF2 gene of the GI.1 Norwalk virus (Thackray *et al.,* 2006), has confirmed invaluable in the identification of

small molecule inhibitors of viral replication (Thackray *et al.,* 2013). Besides, analysis using this method has improved the understanding of the effect of NoV replication on host cell processes, stressing the important role of cholesterol levels on human NoV replication (Pinto *et al.*, 2012).

2.3.1 Clinical Manifestations and Pathology in human

Norovirus infections can lead to diarrhea in some of those who are exposed and are asymptomatic in about one third of the people (Rockx *et al*., 2002). After an incubation period of 10 to 51 hours, the disease often begins with vomiting, trailed by abdominal cramps, fever (in 37 to 45% of the cases), watery diarrhoea, and other constitutional signs such as headache, chills, and myalgias. The disease normally remains only 2 to 3 days but can last longer in nosocomial outbreaks and among children younger than 11 years of age (Rockx *et al.,* 2002). This virus can be secreted in low titers for up to 8 weeks in healthy persons and for more than a year in patients who are immunocompromised. Mortalities have been reported in relation with the outbreaks of gastroenteritis among the elderly in nursing home facilities (Mattner *et al*., 2006). In the United Kingdom, approximately 80 deaths from norovirus infections occur each year among persons older than 64 years of age (Harris *et al*., 2008). Recently, studies suggested norovirus infection is related with necrotizing enterocolitis in new-born, with benign seizures in infants, and with development of inflammatory bowel disease in young patients (Mattner *et al*., 2006).

2.3.2 Clinical Manifestations and Pathology in animal

Noroviruses have related to several non-human species including pigs, cows, sheep, cats, dogs, rats, and mice (Pinto *et al.,* 2012). In many cases where a clinical connection exists, infection typically causes acute gastroenteritis with diarrhoea. The most prominent exception to this is norovirus infection in mice, typically causes subclinical continuous infections (Thackary *et al.,* 2007). Animal norovirus infections have served as models for the learning of human norovirus biology. Furthermore, norovirus-specific antibodies have also been detected in captive juvenile macaques, it is unknown whether infection causes an obvious disease (Farkas *et al.,* 2010). The prototype bovine norovirus was first recognized in 1984 as the causative agent of diarrhea in calves (Karst *et al.,* 2003). More recent studies on GIII.2 bovine norovirus infection of calves showed moderate to severe lethargy followed by acute but continuous diarrhea (Jung *et al.,* 2014). In one study, they found that viral RNA was found in serum for up to 5 days post-infection, indicating viremia, and RNA could be found in faeces for up to 20 days post-infection. The long-term shedding of viral RNA may cause the perseverance of bovine noroviruses in cattle despite the presence of strong antibody reaction (Karst *et al.,* 2003). New-born calves infected with the Jena virus, a GIII.1 bovine norovirus, caused diarrhea and significant conditions of gut pathology, namely severe villous atrophy, continue from 12 hours post-infection until 4 days post-infection (Otto *et al.,* 2011). Murine noroviruses are now documented as widespread pathogens of laboratory mice that in wild-type mice typically caused in long-term sub-clinical persistent infections of the intestine (Arias *et al.,* 2012). Murine

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noroviruses have also been isolated from both field mice and wood mice (Smith *et al.,* 2012).

Furthermore, noroviruses have been isolated from pigs in Japan, the United States, and Europe (Wang *et al.,* 2005). Infection of gnotobiotic piglets with GII porcine noroviruses typically causes mild self-limiting diarrhoea. Gnotobiotic piglets can also be infected with certain genogroup II human noroviruses (Cheetham *et al.,* 2006), becoming a valuable experimental model. Infection of gnotobiotic piglets with a GII.4 human norovirus caused diarrhoea in most animals and led to faecal excretion, seroconversion. The symptomatic nature of infection is particularly related to the testing of candidate therapeutics and vaccines where protection from disease is the most valued read-out. In fact, this study has been applied to prove the efficacy of several human norovirus vaccine candidates (Souza *et al.,* 2007) and several therapeutics (Jung *et al.,* 2012). The current study proved that canine noroviruses use the similar cellular receptors as human noroviruses (Caddy *et al.,* 2014) suggests that transmission of animal noroviruses to humans is theoretically possible. Besides, presence of antibodies to canine norovirus in veterinarians (Mesquiata *et al.,* 2013) signifying that transmission has likely occurred at least in some time.

2.4 Norovirus transmission

In humans, norovirus can be transmitted by consuming food or drink, touch surfaces or objects polluted with norovirus, having direct contact with infected individuals, such as by sharing food or eating utensils (Henry, 2019). Although there are no controlled outbreak studies for both animals and humans, a case of calicivirus

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outbreak in a nursing home in 1983 in the UK was epidemiologically linked to a sick dog.

For animal to human transmission, no animal norovirus has been found in human stool, but some serological evidence proposes the possible transmission of norovirus from animals to humans. Several findings have documented the seroprevalence of bovine and canine (Mesquita *et al.,* 2013) norovirus in humans. Humans that have a close contact and higher exposure to animals have increased titers against animal noroviruses (Widdowson *et al.,* 2005). More veterinarians or porcine specialists had anti-GIII.2 IgG antibodies compared to the control group (28% versus 20%). Similarly, veterinarians showed higher seroprevalence of antibodies to canine GVI.2 VLPs (22.3%) in contrast to control groups (5.8%) (Mesquita *et al.,* 2013). Suggestively, there is no cross-reactivity between human GI.3, GII.1, GII.3, GII.4, GII.6 and bovine GIII.2 was found when convalescent anti-GIII.2 sera of a gnotobiotic calf or specific anti-GIII.2 or GII.3 antibodies were tested. In distinction, studies in Italy showed that cross-reactivity was found between more closely related human GIV.1 and canine GIV.2 noroviruses in an age stratified cohort of 535 people (Mead *et al.,* 1999). In the study, 28.2% of the sera reacted to both GIV.1 and GIV.2 VLPs and only 0.9% detected exclusively GIV.2 VLPs.

For human to animal transmission, many studies have studied the possibility of human norovirus transmission to animals by testing animal stool samples for human noroviruses and by examining the seroprevalence against human norovirus strains. The current outbreak study was one case-control study that included 92 dogs from Finnish

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households. The main inclusion criterion was that either the dog or a human in the household had suffered from diarrhoea and vomiting (Summa *et al.,* 2012). The result showed that 4 dogs were tested PCR positive and all of them originated from households in which at least 2 people suffered from severe gastroenteritis symptoms. From the analyses, 2 GII.4 variants and one GII.12 genotype are detected, 1 GII.4 is like the virus found in the owner's faeces. Seroprevalence against human noroviruses (GI.1, GI.2, GI.3, GII.3, GII.4, GII.6, GII.12) in two dog populations of 1999–2001 and 2012–2013 was studied. The seropositivity against GI and GII VLPs was very low and 10.7–18.6%, respectively (Caddy *et al.,* 2015). Therefore, these studies showed that human noroviruses could infect dogs, but more work is required to prove the potential cross-reactivity with non-human viruses, like GVI.2 (Nakamura *et al.,* 2010).

Several researches in pigs indicated that human norovirus could be detected in pig faeces with more than 1 genotype (Mattison *et al*., 2007). In a study in Japan, the intestinal content of 20 apparently healthy 6-month-old pigs were sampled monthly and upon testing using calicivirus-specific primers, 11 out of 354 were positive for human GII without a seasonal pattern being documented (Nakamura *et al.,* 2010). The strains were classified as GII.4, GII.3 and one GII.13 where all these genogroups were previously reported in an outbreak in humans during that season. In another study in Taiwan on 530 faecal samples of asymptomatic pigs (<8 month) from six farms showed that, 7% tested positive with RdRp-specific primers, while 32% positive samples were positive for GII capsid specific primers. Comparing between season, 41% and 26% positive cases in winter and summer, respectively (Chao *et al*., 2012). In summary, NoV was detected in pigs of all age categories and from different farms.

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Antibodies recognizing human norovirus have been found in healthy household pigs in Nicaragua and US pigs with prevalence ranging from 52%–70% (Farkas *et al.,* 2005). While those antibodies detected VLPs of GI.1, GII.1, GII.3 and GII.4 were not able to block the virus binding pig mucin (Bucardo *et al.,* 2008). During the 2014–2015 epidemic season, GII.17 was the leading human norovirus genotype in some Asian countries. The GII.7 sequences were 99–100% like each other and 95% similar to a human norovirus (KJ196295). Besides, antibodies against various human norovirus genotypes were found in captive primates in the US where antibodies against GI.1, GII.4, GII.5 and GII.7 VLPs were detected in manga beys (85%), macaques (~60–65%), and chimpanzees (92%) (Jung *et al.,* 2014).

There are only few studies of NoV on wild animals compared to livestock animals. Bird faeces were collected for three winters (2009 to 2011) from fresh snow of a household waste dumping site in Finland and were tested positive for GI and GII strains (Summa *et al*, 2012). Of 115 avian faeces, 6 were positive for GI and 25 for GII. Sequencing of four GII.4 (GII.4 2006a/b, 2009) and 2 GII.3 viruses showed that all at least 94% similar to known human NoV strains. Based on cytochrome c oxidase I am sequencing, the positive faeces could be a source of infection to the gulls and crows. Likewise, human norovirus was detected in the intestinal content of a dead Norway rat that had been surviving in the sewer system in Copenhagen (Wobus *et al.*, 2012).

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2.5 Norovirus detection methods

In humans, faecal samples are put in phosphate-buffered solution to a final concentration of 10% (mass/volume). The samples were briefly vortexed, followed by centrifugation at 12,000 rpm for 1 min and supernatant was collected. RNA from fecal samples was extracted using the QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany). Norovirus was detected by reverse transcription (RT)–PCR by amplifying the capsid gene at the C region 21. The amplicon size of the partial capsid gene of GI and GII noroviruses are 330 and 344 bp, respectively. The RNA-dependent RNA polymerase (RdRp) gene was amplified by RT-PCR22. The amplicon size of the partial RdRp gene is 470 bp. The RT–PCR results were confirmed by nucleotide sequencing of the amplicons. The nucleotide sequence of the amplicons was determined by the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions and the product were run on ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The genogroups and genotypes were determined by submitting nucleotide sequences to the Norovirus Genotyping Tool

In porcine, NoV was detected from faecal samples of suckling, weaning and fattening age group (Chao *et al.*, 2012). The samples collection was selected from 6 different farms in swine-farming counties in central and southern regions of Taiwan in 2008. All samples were collected individually from pigs without any gastroenteritis signs. The presumptive positive samples of the PCR products that contained 317-bp amplicons for NoVs were excised from the gel. The products were then purified by the QIAquick gel extraction kit to allow direct sequencing on both strands. To target the

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capsid region, the inner primers G2F3/G2SKR were used for allowing the study of farm animal faeces as a potential pool of NoVs. Same methods as amplifying the RdRp region were used to amplify the capsid region except the annealing temperatures for the primer pairs NV2oF2/NV2oR and G2F3/G2SKR, which were done at 55 °C and 60 °C, respectively. Direct sequencing was performed to confirm the presumptive positive samples were confirmed by. The PCR amplicon recognized as NoV GII.4 infection in humans was utilized as a positive control along with all the PCRs. Primers G2F3/G2SKR were meant to target group 2 norovirus at capsid region, at 5058-5076 nt and 5379-5401nt.

For dog norovirus, similar research was done in Portugal on dogs with diarrhea by screening their faecal sample (Mesquita *et al.,* 2013). Same measures were used for porcine samples. Specific canine norovirus oligonucleotide primers JV102 (5'-TGG GAT TCA ACA CAG CAGAG-3') and JVI03 (5'-TGC GCA ATA GAG TTG ACCTG-3') were chosen for this study. Stool samples from 25 (40%) of the 63 dogs with diarrhea and 4 (9%) of the 42 controls tested positive for a new canine NoVs.

For cat norovirus, there was one research done in Japan on genogroup GVI feline norovirus. Rectal swab samples were taken from cats in an animal shelter. The feline NoV gene was found in samples collected, and it is confirmed by RT-PCR, and these samples were then used in this study. Primers sets P290 and P289d (Pinto *et al.,* 2012), were selected in this research.

Apart from molecular biology, there was one study in Portugal that detected norovirus using serum samples (Mesquita *et al*., 2013). In that study, 510 serum

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samples were collected from dogs that visited veterinary clinics in 14 countries in Europe between September2009 and January 2010.Virus-like particles (VLPs) were produced in Sf9 insect cells infected with a recombinant baculovirus containing the full lengthVP1/VP2 (ORF2 and ORF3 of the genome) of the canine norovirus strain Ca/PT/2007/GVI.2/Viseu (GenBank accession number GQ443611). The morphology and sizes of the VLPs were confirmed by electron microscopy, and the purity was confirmed on Coomassie blue-stained SDS-PAGE gels. All dog sera (n = 510 samples) were tested for IgG antibodies against canine norovirus (GVI.2) using an in-house VLP-based enzyme immunoassay (EIA). Briefly, microtiter plates (Immulon 2HB; Thermo Electron Corporation, Milford, MA, USA) were coated with a purified GVI norovirus VLP (0.25 g/well) in coating buffer (15 mM Na2CO3, 35 mM NaHCO3 [pH 9.6]). After they were washed three times with PBST, the wells were incubated with goat anti-dog IgG-horseradish peroxidase diluted 1:6,400 in blocking buffer for 1 h at 37°C. Bound dog IgG was detected using TMB (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD,USA) at room temperature for 10 min, and the reaction was stopped with stop solution (Kirkegaard & Perry Laboratories,Inc.). The optical density (OD) was measured at 450 nm (MRXRevelation spectrophotometer; Dynex, Magellan BioScience). Serum sample was considered positive when the corrected OD value (OD of VLP-coated wells minus that of the uncoated wells) was above the mean of the ODs from the uncoated wells plus 3 standard deviations. Dog sera for which sufficient volume was left for additional testing (n=30) were also tested for reactivity against human norovirus GI.1 (NC_001959) and GII.4 Minerva (GII.4/DenHaag) (JQ478409.1)

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Comparing detection of norovirus in serum and faecal samples, norovirus prevalence in serum is higher than in faecal detection in a study in the USA (Farkas *et al.,* 2005). In that study, they detected 71% of prevalence rate in porcine by using enzyme-linked immunosorbent assay method to detect the antibodies against norovirus. In the same study, they were using RT-PCR to detect norovirus from stool samples, the prevalence rate by using this method was 2% only. The huge difference of prevalence rate between the two methods is because seroprevalence may indicate past and present exposure to norovirus by detecting the antibodies against norovirus while RT-PCR in faecal detect antigen of norovirus that arise from the shedding of virus during active infection of norovirus in that animal. The animal may be exposed to norovirus before but during the time of detection, the animal may already recover from the virus infection. So, they could not find viral antigen anymore in the faecal because the shedding period of the virus in faecal is about 7 days (Villabruna *et al.,* 2019). The antibodies against norovirus will still present in the blood after the active infection, that is why they detected high prevalence rate of norovirus in seroprevalence study.

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MATERIALS AND METHODS

3.0 IACUC approval

This study involving the usage of animal faecal samples. 24 porcine, 20 canine and 20 feline were proposed to the application IACUC on May 2020. In the application, per rectum faecal collection method was proposed in the application.

3.1 Animal selection

This study consisted of porcine, canine and feline species in which their faecal samples were conveniently sampled and subjected to detection of norovirus. A total of 24 fresh faecal samples each were obtained from porcine and canine through rectal method. A total of 24 archived feline faecal samples were obtained (Appendix 1).

Porcine samples were obtained from two selected pig farms (farm A with closed house management and farm B with open house management) in Selangor, consisting of weaner and sow (Table 3.1).

Table 3.1: Data on the total number of porcine faecal samples from two production stages obtained from two pigs farms used for the detection of porcine norovirus

Farm ID	Production Stage			Total
	Weaner		Sow	
Selangor Farm A	6		6	12
Selangor Farm B	6		6	12
Total	12		12	24

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Canine samples of mongrel dogs were obtained from one animal shelter at Selangor. They consisted of 11 male and 13 females. Age ranges from 1-3 years old with BSC (Body score condition) ranges between 2 and 3 (Table 3.2).

Table 3.2: Data on the canine faecal samples of mongrel dogs obtained from an animal shelter used for the detection of canine norovirus

No.	Code	Gender	Age (Years)	BCS UPON SCORE
1	Dog 1	F	1	3
2	Dog 2	F	3	2
3	Dog 3	F	2	3
4	Dog 4	M	1	3
5	Dog 5	F	5	3
6	Dog 6	F	2	2
7	Dog 7	M	3	3
8	Dog 8	M	3	3
10	Dog 10	F	2	3
11	Dog 11	M	<2	3
12	Dog 12	M	8	3
13	Dog 13	F	1	3
14	Dog 14	F	2	2
15	Dog 15	M	6	3
16	Dog 16	F	2	2
17	Dog 17	M	1	3
18	Dog 18	M	1	2
19	Dog 19	F	2	3
20	Dog 20	F	7	2
21	Dog 21	M	3	2
22	Dog 22	M	2	2
23	Puppy 1	F	<1	2
24	Puppy 2	F	<1	2

Legend- BSC = body score condition; F = Female, M = Male

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Feline samples were archived samples obtained from domestic short hair (DSH) cats originated from one animal shelter in Selangor sampled in the year of 2019. They were composed of 4 males and 16 females. Age ranges from 1-8 years old with BSC ranges between 2 and 3.

Table 3.3: Data on archived feline faecal samples of domestic short hair obtained from an animal shelter was used for the detection of feline norovirus

No.	Code	Gender	Age (Years)	BCS upon score
1	Cat 1	M	8	3
2	Cat 2	F	2	3
3	Cat 3	F	3	2
4	Cat 4	F	1	2
5	Cat 5	F	3	2
6	Cat 6	F	2	2
7	Cat 7	M	1	2
8	Cat 8	F	5	3
9	Cat 10	F	3	3
10	Cat 11	F	1	2
11	Cat 12	M	3	2
12	Cat 13	F	1	2
13	Cat 14	F	2	2
14	Cat 15	F	3	3
15	Cat 16	F	8	2
16	Cat 17	F	1	2
17	Cat 18	F	1	2
18	Cat 19	F	2	2
19	Cat 20	M	2	3

Legend: BSC = body score condition; M male; F female;

3.2 RNA extraction

The RNA extraction was performed using Qiagen QIAamp Viral RNA mini kit (Qiagen, Germany). Briefly, 560 µl prepared Buffer AVL containing carrier RNA was

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pipetted into a 1.5 ml microcentrifuge tube (Eppendoff, Germany). 140 µl of faecal supernatant was transferred to the Buffer AVL–carrier RNA in the microcentrifuge tube. The mixture was mixed by pulse-vortexing (Dragonlab, USA) for 15 seconds. The mixture was left at room temperature for 10 minutes to lyse the faecal particle. The tube was briefly centrifuge (Eppendorf, Germany) to remove drops from the inside of the lid. 560 µl absolute ethanol (ACS Chemical, Malaysia) was added to the sample, and mixed by pulse-vortexing for 15 seconds. After mixing, the tube was briefly centrifuged to remove drops from inside the lid. A total of 630 µl of the solution was carefully added to the QIAamp Mini column in a 2 ml collection tube and centrifuge at 6000 *xg* for 1 min. The QIAamp Mini column is placed into a clean 2 ml collection tube, and the tube containing the filtrate was carefully discarded. 500 µl Buffer AW1 was added into the mini column, and centrifuged at 6000 *xg* for 1 min. The QIAamp Mini column was then placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded. The QIAamp Mini column was added 500 µl Buffer AW2 and centrifuged at full speed 10,000 x*g* for 3 minutes. The QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge and 60 µl Buffer AVE was added and incubated at room temperature for 1 minute. The final product was centrifuged at 10,000 x*g* for 1 minute. The final products were stored in the -80 °C fridge (Panasonic, Japan). (Appendix

3.3 Reverse transcription

The reverse transcription was performed by using a commercial kit (QuantiNova™ Reverse Transcription Kit (Qiagen, Germany). All reactions were performed on ice to minimize the risk of RNA degradation. Additional RNase inhibitors

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or dNTPs were not included in this assay. The QuantiNova Internal Control RNA (QN IC RNA) was an internal amplification control that was used to check successful reverse transcription/amplification. A mixture of RT primers was included in the Reverse Transcription Mix. The RT primer mix is optimized to provide high cDNA yields for all RNA transcript regions. To omit gDNA removal, Reverse-Transcription Master was prepared. The reagent was mixed by combining the Reverse Transcription Mix with the Reverse Transcription Enzyme and adding the mixture into the gDNA Removal Mix.

First, the template RNA, Internal control (QuantiNova Internal control (optional), gDNA Removal Mix and Reverse Transcription Enzyme were thawed on ice. Reverse Transcription Mix and RNase free water were thawed at room temperature (15–25°C). Each solution was mixed by flicking the tubes. The mixtures were centrifuged briefly to collect residual liquid from the sides of the tubes and then keep on ice. The genomic DNA removal reaction was prepared on ice according to table 3.4.

Table 3.4. Genomic DNA removal reaction components for detection of norovirus

Component	Volume/reaction
gDNA Removal Mix	2 µl
Template RNA, up to 5 µg	Variable
Internal Control RNA (optional)	1 µl
RNase-free water	Variable
Total reaction volume	15 µl

The solutions were then mixed and then kept on ice. The genomic DNA removal reactions were prepared on ice. The mixture was mixed and then kept on ice. The mixture was then incubated for 2 min at 45°C, then placed immediately on ice. The Reverse-transcription Master Mix was prepared on ice according to Table 3.5.

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Table 3.5. Reverse-transcription reaction components for detection of norovirus

Component	Volume/reaction
Reverse-transcription Master Mix: Reverse Transcription Enzyme	1 µl
Reverse Transcription Mix	4 µl
Template RNA Entire genomic DNA elimination reaction	15 µl
Total reaction volume	20 µl

The mixtures were mixed and then kept on ice. The Reverse-transcription Master Mix contains all components required for first-strand cDNA synthesis except template RNA. Freshly prepared Reverse-transcription Master Mix was added to each tube containing template RNA from faecal materials of different species of animals. The mixtures were incubated for 3 min at 25°C, 10 min at 45°C and lastly 5 min at 85°C to inactivate the reverse transcriptase enzyme. The mixture was kept at -20C until further used for PCR assay.

3.4 Primer selection

Primers are short pieces of single-stranded DNA used for initiation of DNA synthesis. DNA polymerases are only capable of adding nucleotides to the 3'-end of an existing nucleic acid with the presence of primer bound to the template creating complementary strand. In this study, 4 primers pairs were selected which comprises of one universal primer, and one each for canine, porcine and feline specific primers The universal primers set was obtained from published report (Nakamura *et al.*, 2010), with product of 320 bp (Table 3.6).

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Table 3.6: Universal primers that target RdRP region was used for DNA amplification of norovirus

Primer	Nucleotide Sequence	Primer Location (nt)	Reference
Forward	5' - GATTACTCCAAGTGGGACTCCAC - 3'	4445	Nakamura *et al.* (2010)
Reverse	5'-TGACAATGTAATCATCACCATA- 3'	4763	

For detection of norovirus in porcine, an established primer set with an expected product of 330 bp was used (Kojima *et al.*, 2002) (Table 3.7).

Table 3.7: Porcine specific primers that target capsid region used for DNA amplification of porcine norovirus

Primer	Nucleotide Sequence	Primer Location (nt)	Reference
Forward	5' - CNTGGGAGGGCGATCGCAA - 3'	5058	Kojima *et al.* (2002)
Reverse	5' - CCRCCNGCATRHCCRTTRTACAT - 3'	5401	

For detection of canine norovirus, an established primer set with expected product of 215 bp was used (Mesquita *et al.*, 2013) (Table 3.8).

Table 3.8: Canine specific primers that target capsid region used for DNA amplification of canine norovirus

Primer	Nucleotide Sequence	Primer Location (nt)	Reference
Forward	5' -TGG GAT TCA ACA CAG CAG AG- 3'	3357	Mesquita *et al.* (2013)
Reverse	5' -TGC GCA ATA GAG TTG ACC TG- 3'	3357	

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For feline norovirus detection, an established primer set with expected products of 328 and 312 bp was used (Vennema *et al*., 2002) (Table 3.9).

Table 3.9: Feline specific primers that target capsid region used for DNA amplification of feline norovirus

Primer	Nucleotide Sequence	Reference
Forward	5' - ATACCACCTATGATGCAGAYTA - 3'	Vennema *et al*. (2002)
Reverse	5' - TCATCATCACCATAGAAGAG - 3'	

3.5 Positive control selection

3.5.1 Synthetic oligonucleotide

A synthetic oligonucleotide was constructed with the gene sequence of the highlighted priming sites generating 319 bp amplicon to produce positive control for RT-PCR process based on the universal primer sequence. The multiple sequence alignment was analysed by Clustal Omega (Appendix 2). The construct was commercially produced by Apical Scientific Sdn Bhd Malaysia.

3.5.2 Human Norovirus G1 and G11

Two positive controls for group I and II norovirus in the form of cDNA were kindly donated by Prof Dr Kamaruddin Ahmed, Universiti of Sabah. The synthetic peptide and human positive Norovirus controls were used with PCR assay for detection of NoV RdRp gene by using universal primer pair p290/p289 to produce 319 bp PCR band. Electrophoresis was carried out on 2.0% (w/v) agarose gel. All positive controls and synthetic peptide samples show significant amplification. The result has proved the validity of synthetic peptide and positive controls that we were using in this study.

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3.6 Polymerase chain reaction

Polymerase Chain Reaction (PCR) was used to amplify regions of DNA, generating thousands to millions of copies of specific DNA sequences. In this study, PCR reaction mixture was carried out using MyTaq® Red Mix (Bioline®, US). The mixture contains 10 µl MyTaq® Red Mix, 2 µl of forward primer and 2 µl of reverse primer (20 pmol for universal primer and 10pmol each for species specific primer), 1 µl of RNase-free water, and 5 µl of template, making up a total volume of 20 µl.

PCR amplification was completed with a programmed thermal cycler (T100™ Thermal Cycler, Bio-Rad, USA) involving five steps. It was started with an initial heat activation or pre-denaturation step at 94°C for 5 min to activate the MyTaq® Red Mix (Bioline, US). The PCR reaction conditions included 35 cycles of denaturation at 94°C for 30 seconds to separate the DNA strands providing single-stranded template, annealing at 55°C for 30 seconds to cool the reaction so that primers can bind to their complementary sequences on the single-stranded template DNA, extension at 72°C for 1 min to allow Taq polymerase to extend the primers synthesizing new DNA strands and a final extension at 72°C for 10 min (Table 3.10).

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Table 3.10: Cycling conditions of conventional PCR assay for detection of norovirus using universal primer set

Step	Time	Temperature	Cycle(s)
Initial Heat Activation	1 min	94ºC	1
Denaturation	15 sec	94ºC	35
Annealing	10 sec	50ºC	
Extension	10 sec	72ºC	
Final Extension	1 min	72ºC	1

Source: Scheuer *et al.* (2013).

Table 3.11: Cycling conditions of conventional PCR assay for detection of porcine norovirus using porcine specific primers

Step	Time	Temperature	Cycle(s)
Initial Heat Activation	1 min	94ºC	1
Denaturation	15 sec	94ºC	35
Annealing	10 sec	55ºC	
Extension	10 sec	72ºC	
Final Extension	1 min	72ºC	1

Source: Ayaka *et al.* (2019)

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Table 3.12: Cycling conditions of conventional PCR assay for detection of canine norovirus using canine specific primer

Step	Time	Temperature	Cycle(s)
Initial Heat Activation	1 min	94ºC	1
Denaturation	15 sec	94ºC	35
Annealing	10 sec	37ºC	
Extension	10 sec	72ºC	
Final Extension	1 min	72ºC	1

Source: Mesquita *et al.* (2013)

Table 3.13: Cycling conditions of conventional PCR assay for detection of feline norovirus using feline specific primer

Step	Time	Temperature	Cycle(s)
Initial Heat Activation	1 min	94ºC	1
Denaturation	15 sec	94ºC	35
Annealing	10 sec	50ºC	
Extension	10 sec	72ºC	
Final Extension	1 min	72ºC	1

Source: Vennema *et al.* (2002)

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3.7 Agarose gel electrophoresis and photography

Gel electrophoresis is a technique in which DNA fragments are pulled through a gel matrix that contains small pores by an electric current, whereby DNA fragments are separated according to molecular size. A standard, or DNA ladder, is typically included in order to determine the size of the fragments in the PCR sample. DNA fragments will move towards the positive electrode as they are negatively charged. As all DNA fragments possess the same amount of charge per mass, small fragments can move through the gel faster than large ones. DNA fragments of the same length will form a "band" on the gel, which can be visualized by naked eye.

Two percent (w/v) agarose gel (Vivantis, Malaysia) was prepared for gel electrophoresis by mixing 100 ml of 1x tris-acetate-ethylenediaminetetraacetic acid buffer (TAE buffer) and 2 g of agarose powder (brand, country), dissolved in microwave for about 45 s (Panasonic, Malaysia). The dissolved solution could cool to about 60 ºC and 2.5 µl of nucleic acid staining solution (RedsafeTM, Intron, Korea) was added, followed by gentle swirling of the glass bottle (Schott, Germany) to mix them thoroughly. The agarose solution was then poured into a gel mold with an appropriate well-formed comb to make a gel of 3 – 5 mm thick, and allowed to solidify for about 30 min. Before use, air bubbles were gently removed under or between the teeth of the comb. A small amount of electrophoresis buffer was poured on the top of the gel before carefully removing the comb. The solidified gel was removed from the mold and placed into an electrophoresis tank (Bio-Rad, USA), where a TAE buffer was added until it covered the surface of the gel by approximately 5 mm.

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Five µl of GelPilot 100bp Plus Ladder (Qiagen®, Germany) DNA molecular weight marker was added into the first well and the PCR product from each sample was pipetted and loaded into subsequent wells. The power source (PowerPac™ Basic, Bio-Rad, USA) was set to 120V and allowed to run for about 40 min or until the DNA ladder reached near the bottom end of the gel. Lastly, the gel was placed into the GeneGenius Gel Imaging System (Syngene, India) to be examined through UV illumination. The DNA bands were photographed (GeneSnap 7.1 software Syngene, India) and images were saved for records.

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RESULTS

4.1 Sampling of animals

The sampling of the animals used in this whole study were approved by IACUC, with the reference number of UPM/IACUC/AUP-U021/2020 dated 20 July 2020 (Appendix 3).

4.2 Measurement of RNA concentration and purity

The RNA concentration and purity for all the samples were measured by using Nanodrop 1000 spectrophotometer. The result of RNA concentration and purity are included in (Appendix 4).

4.3 Optimisation of universal primer detecting norovirus on positive controls Human NoVs s group I and group II

A PCR assay for detection of NoV RdRp gene in positive control samples was done by using universal primer pair p290/p289 by Nakamura et al. (2010) to produce 319 bp PCR band. A marker of 100 bp DNA marker was used in this process. Electrophoresis was carried out on 2.0% (w/v) agarose gel. Both samples show specific amplification. This indicated the universal primer pair p290/p289 in our study was able to detect norovirus group I and group II (Figure 4.1).

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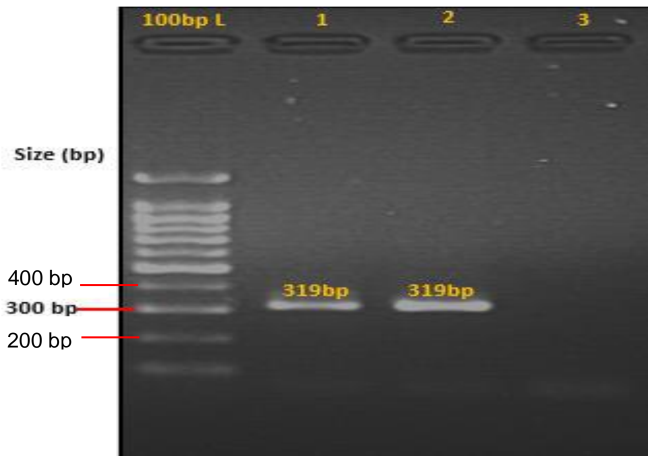

Figure 4.1 PCR assay for detection of NoV RdRp gene in positive control samples by using universal primer pair p290/p289 Both samples NoV GI and GII show specific amplification producing 319bp PCR product

Lane 1: NoV GI positive control as DNA template

Lane 2: NoV GII positive control as DNA template

Lane 3: No template reaction (negative control)

4.4 Detection of Norovirus in multiple animal species by RT-PCR assay using Universal Primers

PCR assay was performed using universal primers by Nakamura *et al*. (2010), with the expected product size 320 bp. For porcine a total of 12 samples comprising 6 weaners and 6 sows were chosen respectively from both farm A and B. None of the samples showed positive amplification (Figure 4.2).

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Figure 4.2 PCR assay for detection of NoV RdRp gene in porcine faecal samples by using universal primer pair p290/p289 to produce 319 bp PCR band. Electrophoresis was carried out on 2.0% (w/v) agarose gel. All samples show no significant amplification

Lane 1: NoV GII positive control (synthetic oligonucleotide)
Lane 2: NoV GII positive control (Courtesy of UMS)
Lane 3: NoV GI positive control (Courtesy of UMS)
Lane 4-15: No bands showed negative result.
Lane NTR: No template reaction (negative control)

For canine, out of 23 dogs' samples, none of the samples showed positive amplification (Figure 4.3).

Figure 4.3 PCR assay for detection of NoV RdRp gene in canine faecal samples by using universal primer pair p290/p289 to produce 319 bp PCR band. Electrophoresis was carried out on 2.0% (w/v) agarose gel. All samples show no significant amplification

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Lane M: 100 bp DNA marker (Qiagen)
Lane 1: NoV GII positive control (synthetic nucleotide)
Lane 2: NoV GII positive control (Courtesy of UMS)
Lane 3: NoV GI positive control (Courtesy of UMS)
Lane 4-15: No bands showed negative result.
Lane NTR: No template reaction (negative control)

For felines, out of 20 samples, none of the samples showed positive amplification (Figure 4.4).

Figure 4.4 PCR assay for detection of NoV RdRp gene in feline faecal samples by using universal primer pair p290/p289 to produce 319 bp PCR band. Electrophoresis was carried out on 2.0% (w/v) agarose gel. All samples show no significant amplification.

Lane M: 100 bp DNA marker (Qiagen)
Lane 1: NoV GII positive control (synthetic nucleotide)
Lane 2: NoV GII positive control (Courtesy of UMS)
Lane 3: NoV GI positive control (Courtesy of UMS)
Lane 4-15: No bands showed negative result.
Lane NTR: No template reaction (negative control)

Out of 24 porcine, all animals were found negative for porcine norovirus antigen following RT-PCR assay, marking a prevalence of 0% (0/24). Out of 23 canines, all animals were found negative for canine norovirus antigen, marking a prevalence of 0% (0/24). Out of 20 felines, all animals were found negative for feline norovirus antigen, marking a prevalence of 0% (0/24). Based on the results above, the prevalence of norovirus in porcine, canine and feline for Selangor was calculated. The prevalence rate

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for porcine farm A and B are 0%. The prevalence rate for animal shelter where the dog and cat faecal samples are collected are 0 % respectively for both species. The total prevalence detected in porcine, canine and feline in this study was found to be 0% (0/67) (Table 4.1).

Table 4.1. Prevalence rates of norovirus in porcine, canine and feline from farms and animal shelters in Selangor following RT-PCR assay by using universal primer on faecal samples

Locations	Age Group	Total no. of animal (n)	Norovirus		Positive Rate (%)		
			Positive (n)	Negative (n)	Individual Farm	Species	States
Porcine Farm A	Weaner	6	0	6	0/12 (0)	0/24 (0)	0/67 (0)
	Sow	6	0	6			
Porcine Farm B	Weaner	6	0	6	0/12 (0)		
	Sow	6	0	6			
Dog shelter		23	0	23	0/23 (0)	0/23 (0)	
Cat shelter		20	0	23	0/20 (0)	0/20 (0)	

n = number of samples

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Furthermore, Fisher's Exact test was also carried out to determine if there is any significant association between the norovirus prevalence rate and age group of porcine (weaner and sow). A significant association is deemed only if p-value ≤ 0.05. In this study, we do not have positive results for Norovirus PCR assay detection results. Therefore, we cannot prove the association between norovirus prevalence rate and age group of porcine (Table 4.2).

Table 4.2 Table shows the frequency data of positive Porcine Norovirus (NoVs) in the faecal sample with different age groups. (Norovirus was not detected in the opened and closed house management system porcine farm)

Age Group of Animal		Detection of NoVs by RT-PCR		
		NoVs+ (n)	**NoVs- (n)**	**Total (n)**
	Weaner	0	12	12
	Sow	0	12	12
Total		0	24	24

4.5 Detection of Norovirus in porcine, canine and feline faecal samples by using RT-PCR assay using species specific primers

Specific primer that we prepared was not used in this study because Norovirus was not detected in porcine, canine and feline samples by using universal primer.

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DISCUSSION

This study is a pilot study to determine the status of norovirus in porcine, canine and feline in Malaysia. However, this study is not able to demonstrate any positive norovirus antigen in porcine, canine and feline from the animals by using their faecal samples. It is acceptable to obtain negative results from tested animals because the virus may not present in the selected farms and animal shelters in Selangor. There are several possibilities regarding negativity of norovirus in the animals observed in this study.

First, norovirus may be absent in faecal samples of porcine, canine and feline in Selangor. Norovirus has been detected in faecal samples of humans in several studies in Malaysia (Kamruddin *et al.*, 2020) but no study has been done in animals in Malaysia. There is a lack of baseline data of the virus status in animals in Malaysia as this is the first study done on multiple animal species. From other studies in the Asia region, the detection rate of viruses in animals is lower compared to the study of norovirus in humans (Chao *et al.,* 2012; Nakamura *et al.,* 2010). Therefore, it is highly possible that norovirus is absent in the faecal sample of porcine, canine and feline in Selangor.

Besides, the isolation of high-quality RNA from stool is a challenge. The presence of humic acid compounds, fats and other bio by products in stool makes the isolation of quality nucleic acid samples that are free of PCR inhibitors very challenging. Furthermore, the presence of RNases and DNases in stool poses a logistical problem in the form of nucleic acid degradation that occurs during sample collection and transport (Sonke *et al.*, 2019). In this study, the RNA concentration and purity were measured by Nanodrop each time the sample was extracted to ensure the purity of the RNA template. Overall, the

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RNA concentration and purity obtained are not optimum as the RNA has degraded in most of the samples during the extraction process. The resultant RNA concentrations are considered low amount and purity, and this may affect the yield of cDNA from the reverse transcription process as less RNA is available for the cDNA conversion.

There was also study testing norovirus seroprevalence rate by using serum samples. In that study, the seroprevalence rate is higher compared to faecal antigen detection method. They detected 71% of norovirus prevalence rate by using serum samples from porcine while by using faecal samples, they only got 15% prevalence rate (Farkas *et al.*, 2005). The higher detection rate in serology testing because seroprevalence detects the antibodies against norovirus, where the antibodies may be produced by the body from previous infection and current infection. While for faecal antigen detection, this method only detects those animals under active infection, where the animals will shed the virus antigen during the infection. During acute infection, the animals' body immune system will respond by producing antibodies against the virus. The antibodies will neutralize the virus antigen and eventually fight off the infection. When the animal is recovered and out of infection stage, the virus genome detection in the faecal samples may appear to be negative because the animal already stopped shedding the virus. However, the antibodies from the previous infection will still be present in the animal body as some of the B immune cells will become memory immune cells that will last long in the circulation. The current study is targeting active infection animals instead of past infection, and thus faecal antigen detection methods were suitable for the study.

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Besides, in one study researcher found that norovirus will shed in the faecal for an average of 5 days (Lou *et al.,* 2015). During this active infection, the animal will shed the virus. When the shedding period passes, the animal will not shed the virus anymore. Therefore, no virus antigen will be detected at the faecal samples by using molecular methods after the virus shedding period. Probably there is a low copy virus that was shed but was able to be detected by the current assay method. In order to overcome the short shedding period, future study should be conducted on detecting the antibodies to assess the seroprevalence of norovirus in animals. Besides, the samples collected in this study are faecal samples from clinically healthy animals. In one study, researchers found that diarrhea patients have a higher prevalence rate compared to healthy patients when they detect norovirus from faecal samples (Sokel *et al.,* 2019). Therefore, the detection rate for norovirus will be lower compared to other studies due to the different health status of the host. Diarrhea animals are in an active infection state and will shed more virus during the infection stage (Sokel *et al.,* 2019). In future study, parameters should include the diarrhea samples by collecting it over time to increase the detection rate of norovirus.

Furthermore, for NoVs, it was found to slightly increase in the winter season. For porcine NoVs, there is one study found that the winter season has a higher virus detection rate compared to summer (Morimitsu *et al.,* 2014). In another study in the United States, the study concluded that epidemics purportedly had occurred on a particular farm during a particular month especially during winter season, as the viruses were not detectable through surveys several months later during summer season (Wang *et al.*, 2005). Same to the winter seasonality in human NoVs that causes in epidemics in different close settings (O'Neill *et al.,* 2005), a seasonal trend was also detected in swine with 26.4%

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and 41.7% of the positive rate during the spring and winter, respectively, with statistical significance ($P < 0.05$). The studies that have successfully detected norovirus are mainly conducted in temperate and sub temperate countries (Nakamura *et al.*, 2010; Chao *et al.,* 2012). Malaysia is in a tropical region which has higher temperature compared to temperate countries so the prevalence of norovirus in Malaysia may be lower compared to temperate countries due to unfavorable environment for the virus survival. For this pilot study, porcine samples are obtained from 2 different environment settings to imitate in temperate country weather. Farm A is a closed house management farm where the sows, and weaner are kept indoors with an air-cooling system of ≤25 degree Celsius. However, this is not enough to imitate temperate country weather, where during winter season, the temperature can down until below 0-degree Celsius, which could favor the virus survival in the environment.

Besides, the sample size for study may be too small for this study compared to other published reports. There was a study in Taiwan that investigated the epidemiological features and genotypes of caliciviruses in swine farms using 533 pig faecal samples from six farms in central and southern Taiwan. The NoVs were detected with a positive rate of only 7.1% (Chao *et al.*, 2012). In current study, there are only 20 samples from the swine farm and animal shelter from each species. Due to the nature of the virus being low prevalence in several hosts reported elsewhere, this study might miss some of the positive cases in the total population. Specific sampling methods such as simple random sampling and stratified sampling method, could be used in future study to ensure that all samples are selected randomly and equally to increase the chance of norovirus detection.

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This study concluded that the porcine, of weaner and sow age group, and canine and feline in an animal shelter in Selangor are free from porcine norovirus. However, the sample size is too small, and the selected farms are not enough to represent farms or animal shelters in Selangor. As this is a pilot study, this study provides few suggestions for future study on animal's norovirus in Malaysia.

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CONCLUSION

Despite the world-wide norovirus detection, there is still no data on norovirus on porcine, canine and feline in Malaysia. In this study, we want to know whether Norovirus present in healthy porcine, canine and feline in Selangor. Besides, we want to know the prevalence rate of Norovirus in animals of Selangor. From this study, Norovirus was not detected in porcine for weaner and sow age groups in farms in Selangor via conventional RT-PCR assay. Similarly, Norovirus was also not detected in canine and feline in animal shelters in Selangor via conventional RT-PCR assay. In future study, diarrhea samples and serological study can be chosen to detect norovirus.

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RECOMMENDATIONS

Norovirus is not able to be detected in the faecal sample in porcine, canine and feline in Selangor. There are several recommendations for future studies.

First, the sample size could be increased. Since the reported prevalence rate of norovirus is low, future study should increase the sample collection from different farms in Malaysia to increase norovirus detection rate.

Besides, samples from cases of diarrhea samples of animals ought to be used. Animals in the active infection stage normally have diarrhea symptoms, have higher chances of detecting the virus genome when the animals are shedding the virus.

Furthermore, the diagnostic method could be improved by using real time PCR to increase the detection rate. This is because real time PCR is able to detect low quantity of targeted RNA compared to conventional RT-PCR. Therefore, this method will useful to detect the presence of norovirus that has low detection rate.

Lastly, to perform seroprevalence study to detect post exposure of animals to the virus to prove the animals have exposure to the virus in their lifetime.

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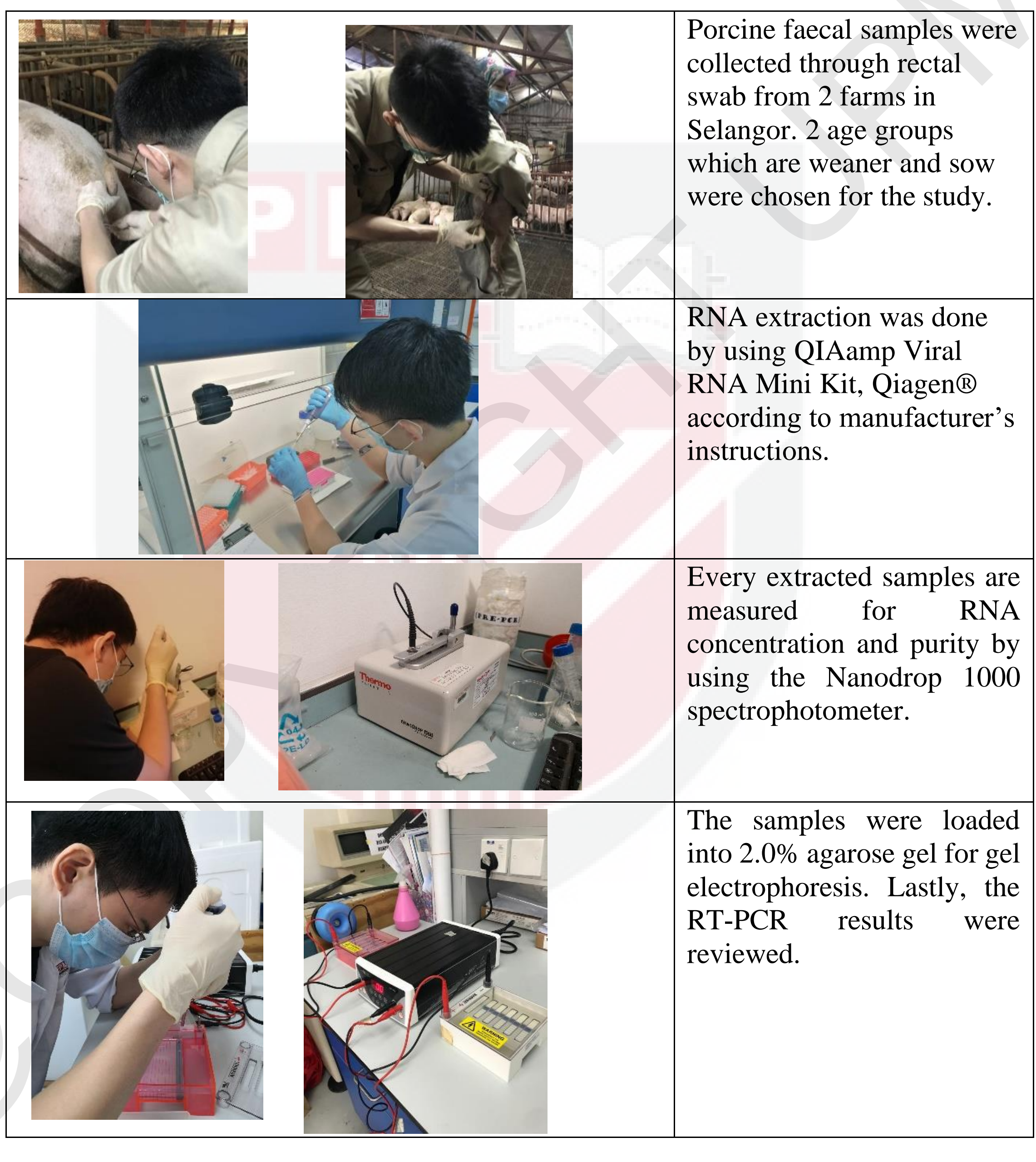
APPENDICES

Appendix 1

Pictures of samples collection, processing and results reviewing



Pictures	Description
	Porcine faecal samples were collected through rectal swab from 2 farms in Selangor. 2 age groups which are weaner and sow were chosen for the study.
	RNA extraction was done by using QIAamp Viral RNA Mini Kit, Qiagen® according to manufacturer's instructions.
	Every extracted samples are measured for RNA concentration and purity by using the Nanodrop 1000 spectrophotometer.
	The samples were loaded into 2.0% agarose gel for gel electrophoresis. Lastly, the RT-PCR results were reviewed.

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Appendix 2

Design of Norovirus Gene Construct

Gene sequence to be constructed with highlighted priming site generating 319 bp amplicon:
5'GATTACTCCAAGTGGGACTCCACACAACAAAGAGCCGTGTTGGCAGCAGC
CCTAGAAATCATGGTTAAATTCTCCTCAGAACCACACTTGGCTCAAGTAGTC
GCAGAAGACCTTCTTTCTCCTAGCGTGGTGGATGTGGGTGACTTCAAAATAT
CAATCAATGAGGGTCTCCCCTCTGGGGTGCCCTGTACCTCCCAATGGAACTC
CATCGCCCACTGGCTTCTCACTCTCTGTGCGCTCTCTGAAGTCACGAACTTG
TCCCCTGACATCATACAGGCTAACTCCCTCTTCTCCTTTTATGGTGATGATTA
CATTGTCA 3'
Size: 319 bp
Purpose: To serve as a positive control in RT-PCR detection (diagnostic test)
Primer pair that are being used in current project:

Primer	Sequence	Size (bp)	Polarity	Nucleotide position in NoV Norovirus GII isolate Hu/GII.P4/HSP279/1999/BRA RNA-dependent RNA polymerase gene, partial cds Accession no. KX702079.1
p290	5'GATTACTCCAAGTGGGACTCCAC	23	Positive	1-23
p289	5'TGACAATGTAATCATCACCATA	22	Negative	298-319

Multiple sequence alignment analysis by Clustal Omega.
RCp289 = Reverse complement of p289

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```
RCp289          ------------------------------------------------------------ 0
NoVKX702079.1   GATTACTCCAAGTGGGACTCCACACAACAAAGAGCCGTGTTGGCAGCAGCCCTAGAAATC 60
p290            GATTACTCCAAGTGGGACTCCAC------------------------------------- 23

RCp289          ------------------------------------------------------------ 0
NoVKX702079.1   ATGGTTAAATTCTCCTCAGAACCACACTTGGCTCAAGTAGTCGCAGAAGACCTTCTTTCT 120
p290            ------------------------------------------------------------ 23

RCp289          ------------------------------------------------------------ 0
NoVKX702079.1   CCTAGCGTGGTGGATGTGGGTGACTTCAAAATATCAATCAATGAGGGTCTCCCCTCTGGG 180
p290            ------------------------------------------------------------ 23

RCp289          ------------------------------------------------------------ 0
NoVKX702079.1   GTGCCCTGTACCTCCCAATGGAACTCCATCGCCCACTGGCTTCTCACTCTCTGTGCGCTC 240
p290            ------------------------------------------------------------ 23

RCp289          ---------------------------------------------------------TAT 3
NoVKX702079.1   TCTGAAGTCACGAACTTGTCCCCTGACATCATACAGGCTAACTCCCTCTTCTCCTTTTAT 300
p290            ------------------------------------------------------------ 23

RCp289          GGTGATGATTACATTGTCA- 22
NoVKX702079.1   GGTGATGATTACATTGTCAA 320
p290            -------------------- 23
```

Appendix 3

IACUC Approval Letter

PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date:	08th July 2020
AUP No.:	UPM/IACUC/AUP-U021/2020
Project Title:	Molecular Detection of Norovirus in Porcine, Canine and Feline in Selangor, Malaysia
Principal Investigator:	Professor Dr. Siti Suri Binti Arshad
Members:	Assoc. Prof. Dr. Ooi Peck Toung, Assoc. Prof. Dr. Gayathri Thevi Selvarajah, Tan Wei Yang
Attending Veterinarian:	Assoc. Prof. Dr. Ooi Peck Toung
Committee Decision:	The committee has reviewed and approved the proposed animal utilisation protocol, subject to relevant permit and/ or owner's consent.
Project Classification:	Acute
Category of Invasiveness:	B
Source of Animals:	1. Pig Farm A, (Grace Agri Farm), Ladang Tumbok, Tanjung Sepat, Selangor 2. Pig Farm B, (Profeed Farm), Batu Laut, Tanjung Sepat, Selangor 3. Pig Farms C, (Yap Ah Lean Farm), Batu Laut, Tanjung Sepat, Selangor
Number of Animals Approved:	20 Pigs, 20 Cats, 20 Dogs
Housing:	Not Applicable
Duration:	10th August 2020 – 31st December 2020

Ethical approval is required in the case of amendments to the approved animal utilisation protocol (AUP). Please apply using Form 105. Kindly submit a final/annual report (Form 106) upon study completion, or before expiry of approval.

PROF. DR. ABDUL RAHMAN OMAR
Chairman
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (P&I) 603-8947 1002 603-8946 4134, Pejabat Pentadbiran TNCPI 603-8947 1608, Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) 603-8947 1601, Pejabat Pengarah, Putra Science Park (PSP) 603-8947 1291
http://www.tncpi.upm.edu.my

Appendix 4

RNA concentration and purity extracted from faecal samples as determined by Nanodrop 100 spectrophotometer.

A. **A total of 20 RNA samples from Porcine**

Samples ID	Concentration mg%	260/280	260/230
AW1	29.4	2.11	0.84
AW2	18.7	2.18	0.72
AW3	41.1	2.25	0.19
AW4	32.1	2.06	1.06
AW5	38.6	2.08	1.43
AW6	29.2	1.93	0.39
AS1	13.7	1.72	0.08
AS2	34.1	1.94	0.8
AS3	11.3	2.13	0.09
AS4	19.4	1.81	0.81
AS5	31.8	1.45	0.24
AS6	34.8	1.57	0.5
BW1	57	1.85	1.49
BW2	17.2	1.96	1.1
BW3	18.2	1.51	0.18
BW4	11.2	2.14	0.09
BW5	40.24	2.04	0.27
BW6	13.08	1.88	1.22
BS1	24.5	1.77	0.18
BS2	15.5	1.89	1.33
BS3	10.55	1.79	0.44
BS4	11.5	1.77	0.11
BS5	8.7	1.59	0.16
BS6	12.6	2.25	0.07
EW6	59	2.15	0.51

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B. A total of 20 RNA samples from Canine

Samples ID	Concentration mg%	260/280	260/230
P1	5.4	2.45	0.33
P2	52.1	2.17	0.23
D1	122.3	2.14	1.12
D2	122.5	2.1	1.6
D3	25.5	2.32	0.18
D4	23.6	1.86	0.14
D5	38.8	2.05	0.21
D6	12.6	2.09	0.71
D7	45.8	1.96	1.47
D8	40.1	2.05	0.16
D10	34	1.96	0.19
D11	47.7	2.01	0.18
D12	68	1.93	0.4
D13	30.8	1.84	0.29
D14	27.5	1.77	0.81
D15	50.2	1.97	0.63
D16	14.33	2.15	0.47
D17	64.83	2.02	1.48
D18	101.88	2.03	2.23
D19	70.4	2.1	0.45
D20	206	2.02	1.46
D21	137.8	2.05	0.87

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C. A total of 20 RNA samples from Feline

Samples ID	Concentration mg%	260/280	260/230
C1	172.3	2.14	1.12
C2	192.5	2.1	1.6
C3	125.5	2.32	0.18
C4	323.6	1.86	0.14
C5	87.9	2.05	0.21
C6	112.6	2.09	0.71
C7	145.8	1.96	1.47
C8	240.1	2.05	0.16
C10	340.1	1.96	0.19
C11	457.7	2.01	0.18
C12	168.7	1.93	0.4
C13	230.8	1.84	0.29
C14	127.5	1.77	0.81
C15	1150.2	1.97	0.63
C16	124.33	2.15	0.47
C17	69.73	2.02	1.48
C18	171.88	2.03	2.23
C19	270.4	2.1	0.45
C20	506	2.02	1.46

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