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Short Communication

Mice as Small Animal Model for *Human Norovirus* Infection

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ABSTRACT

Despite being discovered in 1972, the development of vaccines to prevent *Human Norovirus* (HuNoV) infection in humans remains limited. This limitation is partly due to inadequate knowledge about *Norovirus* infection in non-human hosts and its pathogenic process. This research was performed to assess the efficacy of HuNoV infection in mice as an animal model by using both different infection routes and virus titers. In this research, 20 BALB/c *Mus musculus* were used as the test animals which were randomly divided into 4 groups. Intraperitoneal and oral infection methods were employed using different viral titers. The mice were euthanized on the fifth day to obtain fecal samples from the colon organ. Afterwards, Quantitative Polymerase Chain Reaction (qPCR) was administered to evaluate these samples. Most of the mice did not show symptom, except one mouse that experienced diarrhea. The HuNoV virus replication in the mice was measured using qPCR analysis. Three of the twenty mice showed elevated virus titers after being infected with HuNoV. In conclusion, BALB/c strain mice remained resistant to HuNoV infection when exposed to the virus through the oral route. Conversely, the use of high viral titers for intraperitoneal infection indicated the presence of viral replication.

***Keywords*:** *Norovirus; Animal Model; Mice; Diarrhea*

Introduction

Norovirus is one of the viral pathogens that warrants significant attention as it causes non-bacterial gastroenteritis disease outbreaks in all age groups.[1,2] More than 1 million deaths are attributed to *Norovirus* infection, with children under five years old being particularly vulnerable, accounting for approximately 200,000 fatalities each year.[3] The primary mode of transmission for the virus is the fecal-oral route, which involves ingesting contaminated food or direct contact with infected individuals. [4] Norovirus is a single-stranded positive-sense RNA virus that belongs to the Caliciviridae family.[5,6] The virus has 180 molecules of viral capsid protein 1 (VP1) with an icosahedral capsid structure.[7,8] Its genome length is approximately ~7.6 kb with 3 open reading frames (ORFs). The virus is grouped into five genogroups; GI, GII, GIII, GIV, and GV. The HuNoV belongs to the genogroups GI, GII, and GIV.[9]
Vaccination has been considered an effective intervention that helps prevent the disease's transmission.

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The development of *Norovirus* vaccine requires an animal model to understand the mechanism of *Norovirus* infection in living beings. Wobus *et al.*[10] suggested that using the HuNoV virus or a virus with genetic similarities could improve the development of animal models[10] allowing for the manifestation of the biological and clinical features of HuNoV infection in animal models.
To date, animal model specifically designed for HuNoV is still limited. The first small animal model was deployed in 2013 consisting of BALB/c strain mice deficient in the recombination activation gene (Rag-/-) and the common gamma chain (γc-/-). These mice were grafted with human CD34+ hematopoietic stem cells. Several other animals such as *Gnotobiotic calves*,[11] *Gnotobiotic pigs*,[12–18] *Rhesus macaque*,[19–21] and Chimpanzee[22] had also been used as test animals in the experiment, yet these animals did not show all aspects of HuNoV infection. In this current research, mice were infected with HuNoV to examine the viral replication mechanism.

Materials and Methods

HuNoV Virus Samples
HuNoV samples were obtained from the Institute of Tropical Disease Universitas Airlangga and stored at -80º C. HuNoV titers were measured using qPCR specific for GII. After that, the virus strain was identified by sequencing the RdRp (ORF1) and ORF ½ junction regions.[21] HuNoV suspensions were prepared in a phosphate-buffered saline (PBS) solution at 10% w/v and clarified using a centrifuge. The generated HuNoV samples were then analyzed using qPCR to identify the genomic titer used to infect all of the mice.

Experimental Animals
All treatments conducted in this research had been approved by the Universitas Nahdlatul Ulama Surabaya Medical Research Ethics Committee (No: 0207/EC/KEPK/UNUSA/2022). A total of 20 male

BALB/c strain mice of 3 month age weighing 150-250 grams were obtained from the Institute of Life Sciences, Engineering, and Technology Universitas Airlangga. Samples were then randomly divided into 4 groups; P1 (per-oral infection route + virus titer 10^4), P2 (per-oral infection route + virus titer 10^5), P3 (intraperitoneal infection route + virus titer 10^4), P4 (intraperitoneal infection route + virus titer 10^5). Prior to the experiment, all samples underwent a one-week acclimatization period with unrestricted access to food and water. In this research, fecal sample of each mouse was collected and analyzed using the Rapid Diagnostic Test (RDT) *Norovirus* Kit. The findings of the test indicated that all mice tested negative for *Norovirus*.

Mice Infection Procedure

Before infection, each mouse was tested for Norovirus using a Rapid Diagnostic Test (RDT) to confirm that they test negative for Norovirus. Two routes of infection through oral and intraperitoneal routes of infection were administered to infect the mice. The virus injection dose was 1 ml of HuNoV suspension for all groups of mice, while HuNoV suspensions were injected into mice at concentrations of 10^4 and 10^5 through both routes of administration.

Stool and Organ Sampling

On the 5th day after infection, all mice were anesthetized with a 0.05 mL ketamine injection via the intraperitoneal route. Surgery was performed with a transverse incision in the lower center of the abdomen towards the superior, then the diaphragm was released for blood collection from the cardiac using a 1cc syringe. The blood was then stored in EDTA bottles. After that, the spleen was detached from its surrounding tissues, followed by the removal of the ileum, which was sectioned from the ileocecal junction to approximately 2-3 cm proximally. Fecal samples were extracted from the descending and transverse colon sections. The ileum and spleen tissues were put into Eppendorf bottles containing 10% formalin solution, while the colon tissue was stored in bottles with DMEM solution. All tissues collected were stored at -80ºC. Organ and blood samples taken from mice were not examined and were donated to the Tropical Diseases Center, Universitas Airlangga for further investigation.

qPCR Examination to Determine HuNoV RNA Viral Load

Fecal samples were put into the container bottles and stored at -80ºC. A total of 20 samples were collected and examined for Norovirus using quantitative PCR (qPCR) to detect Genogroup II (GII). The qPCR analysis was performed based on the protocols from earlier investigations.[1,23] A 10% suspension of feces from each sample in distilled water was centrifugated at 21,130 x g for 10 minutes. The primer and probe sets used in this research are shown in Table 1.

Table 1: qPCR primers used for *Human Norovirus* (HuNoV) GII Screening

Genogroup	Primer of probe	Sequence (5'→3')[a]	Polarity[b]	Location
GII	Primer COG2F	CARGARBCNATGTTYAGRTGGATGAG	+	5003
	Primer COG2R	TCGACGCCATCTTCATTCACA	-	5100
	Prober RING-TP	FAM-TGGGAGGGCGATCGCAATCT-TAMRA	+	5048

Results and Discussion

Virus titers of 10^4 and 10^5 were infected to the mice through oral and intraperitoneal routes. Rapid Diagnostic Test (RDT) was performed 48 hours post-infection, with positive results found in 10 samples even though the results were faint. However, it was not directly concluded that all samples were infected with *Norovirus* due to low sensitivity of the measurement instrument.[24] No clinical symptoms, such as diarrhea and vomiting occurred 24 hours post-infection to day 4 post-infection. Behavioral changes were not identified at all during the experimental period. On the 5th day, all mice were anesthetized using a 0.05 mL ketamine injection via the intraperitoneal route. Stool samples can be directly from the colon, blood, and some organs such as the spleen, ileum, and colon. However, in this research, fecal samples were obtained directly from the intestine, considering that the feces in the intestine were not contaminated by the external environment or mixed with other feces. Based on the observation, one mouse that was infected via the intraperitoneal route with a viral titer of 10^5 showed signs of diarrhea, yet the ones in other groups did not show any significant signs. A qPCR assay was then performed to detect viral load in all fecal samples. Of the 20 stool samples, 3 samples showed higher viral titers; P3.1 (3.4 x 10^7), P3.2 (8.6 x 10^7), and P4.1 (5.1 x 10^8) (See Table 2). From these results, the increase in viral titer only occurred in the group infected through intraperitoneal route.

This research describes the optimal route of infection for HuNoV in mice. Animal models for HuNoV are significant in the pathogenesis, biology, and vaccine development. To date, the development of animal models using various species of laboratory animals for HuNoV infection has not successfully replicated the clinical and pathological features characteristic of HuNoV infection in humans.[21,25] Wobus *et al*.[10] explained that the use of mice the test animals is more cost-efficient, with high breeding rates and ability to genetically manipulate the host.

The results of this research differ from the ones obtained by Taube *et al*.[26] who used RAG/γc-/- BALB/c mice. In this present research, BALB/c mice were infected through two different routes using different viral titers. This research shows that HuNoV virus can replicate in mice, yet its viral replication mechanism in mice is not yet explored. In this research, HuNoV virus replication only occurred in the group of mice infected through intraperitoneal route. The findings also revealed an increase in the number of virus titers compared to the initial virus titers used for infection. This finding is contradictory to the findings of Taube *et al*.[26] who did not find HuNoV genome in the fecal analysis of mice infected only through the intraperitoneal route. Furthermore, the findings are in stark contrast to the typical fecal-oral transmission route of HuNoV in humans.[27]

Within the 5-day observation, the mice infected through oral route did not show any symptoms. This finding shows that oral route of infection is not effective route of HuNoV infection in mice.[10] Taube *et al*. (2013) compared the complete sequence of an inoculated human fecal sample and the genome sequence collected from mouse feces 48 hours post-infection and identified the presence of HuNoV in the feces of mice that did not undergo extensive adaptation.[26]

Furthermore, several hindering aspects occurred during this research. First, on the 5th or last day of observation, only three mice showed higher HuNoV viral load. The mice might have naturally recovered from HuNoV infection due to the inherent self-limiting characteristics of the virus.[27] To overcome this issue, future researchers are encouraged to conduct post-infection examination of fecal samples. The experimental animals used in this were BALB/c strain mice rather than "humanized" mice. Bryda (2013) suggested that "humanized" rats might serve as better models for developing human disease treatments.[28]

Table 2: Summary of observation and qPCR assay results

Group	Mice	Infection Route	Infection Doses	Virus replication (genome copies/g stool)	Diarrhea
P1	P1.1	Per-oral	1ml x ($1x 10^4$)	ND*	ND
	P1.2			ND*	ND
	P1.3			ND*	ND
	P1.4			ND	ND
	P1.5			ND	ND
P2	P2.1	Per-oral	1ml x ($1x 10^5$)	ND*	ND
	P2.2			ND*	ND
	P2.3			ND	ND
	P2.4			ND	ND
	P2.5			ND	ND
P3	P3.1	Intraperitoneal	1ml x ($1x 10^4$)	$3,4 x 10^7$*	ND
	P3.2			$8,6 x 10^7$*	ND
	P3.3			ND*	ND
	P3.4			ND	ND
	P3.5			ND	ND
P4	P4.1	Intraperitoneal	1ml x ($1x 10^5$)	$5,1 x 10^8$*	Liquid Diarrhea
	P4.2			ND*	ND
	P4.3			ND	ND
	P4.4			ND	ND
	P4.5			ND	ND

* = Indicated positive through Rapid Diagnostic Norovirus Test Kit examination
ND = Not Detected

Conclusion

In summary, BALB/c mice can be used as an animal model for sub-clinical HuNoV infection, although further research is needed to elucidate the mechanism of viral replication and infected organs. Animal models are crucial in the research and development of vaccines or drugs. This research provides valuable new insights into animal models for HuNoV infection, identifying the Mus musculus strain BALB/c as a potential model. This is characterized by an increase in the viral genome. However, the infection observed in BALB/c mice does not replicate all aspects of human Norovirus infection, such as fecal-oral transmission.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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