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Rotavirus is associated with decompensated diarrhea among young rhesus macaques (*Macaca mulatta*)

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Abstract

Diarrhea with secondary decompensation is the main cause of morbidity and mortality in captive young rhesus macaque (*Macaca mulatta*) colonies. Approximately 25% of diarrhea cases with secondary decompensation are considered to be idiopathic chronic diarrhea. The purpose of this study was to investigate the suspected but not systematically examined association between rotavirus infection and diarrhea with secondary decompensation among young rhesus macaques at the California National Primate Research Center (CNPRC). Blood and stool samples were collected from 89 randomly selected young animals (age range: 6 months to 1.5 years) and were tested for the presence of rotavirus antibody, and rotavirus antigen, respectively, using enzyme-linked immunosorbent assays (ELISA's). Test and clinical data were analyzed using Fisher's exact tests and multivariate logistic regression model. Our analysis indicates that rotavirus is endemic among young outdoor-housed rhesus macaques at the CNPRC. Although the relationship between detectable rotavirus antigen in stool and symptomatic diarrhea with secondary decompensation was not significant, there was a significant association between rotavirus seropositivity and a history of diarrhea with secondary decompensation within the past 6 months. While our cross-sectional and case-control study suggests an association between rotavirus infection and diarrhea with secondary decompensation among captive rhesus macaques, more extensive longitudinal studies on larger cohorts and with more intensive sample collection are needed to confirm these findings.

Keywords

idiopathich chronic diarrhea; ICD; enterocolitis; colony management; gastroenteritis; Colony Management

INTRODUCTION

Diarrhea, which can range from mild and transient to severe and even fatal, is one of the most common symptoms for a multitude of diseases in humans and animals. Based on a 2015 study, globally approximately 10.5% of deaths in children less than 5 years of age were due to diarrhea (Kovacs et al., 2015). Rotavirus infection is considered to be the primary etiology of severe diarrhea in children causing hospitalizations and deaths (Kawai, O'Brien,

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Goveia, Mast, & El Khoury, 2012). Thus, due to the health importance of rotavirus, routine immunizations are recommended by the World Health Organization.

Diarrhea is also a common cause of morbidity and mortality in captive colonies of macaques. Up to 15–39% of outdoor-housed rhesus macaques, especially younger animals, require treatments due to diarrhea (Prongay, Park, & Murphy, 2013). Some pathogenic bacteria, including *Campylobacter, Yersinia, Shigella, and Salmonella* are considered to be the most common pathogens found in cases of diarrhea in rhesus macaques (Blackwood, Tarara, Christe, Spinner, & Lerche, 2008). At the California National Primate Research Center (CNPRC), it is estimated that annually up to 15% of young (≤ 1.5 years of age) outdoor-housed rhesus macaques require hospitalization (defined as temporarily relocating animals indoors for treatment) due to diarrhea with secondary decompensation (i.e., dehydration and electrolyte loss). While some of these diarrhea cases are diagnosed with pathogenic bacteria or parasites, approximately 25% of diarrhea cases are considered to be idiopathic chronic diarrhea (ICD; i.e., diarrhea cases without identification of pathogenic bacteria, parasites or other specific causes) (Ardeshir et al., 2013). Although, there are remarkable amount of evidence that non-human primates are very susceptible to many different types of enteric viruses, thus far, viral etiologies have not been excluded as part of the routine diagnosis(Karlsson et al., 2015). Consequently, the relationship between diarrhea in young rhesus macaques and rotavirus infection is yet unproved and remains unclear.

Furthermore, research on the relationship between rotavirus infection and diarrhea in young macaques is related not only to the captive colonies but also to those wild nonhuman primate colonies. Based on a survey conducted by CNPRC, there is 59% (95/160) of the wild caught monkeys from Africa (including 7 different species: *Chlorocebus aethiops, Cercopithecus albogularis, Papio anubis, Lophocebus albigena, Cercopithecus neglectus, Lophocebus aterrimus, Colobus guereza*) are rotavirus seropositive (Otsyula et al., 1996). Hence, understanding the role of rotavirus infection in the wild environment can be very valuable for future studies on infectious diseases and conservation among wild nonhuman primate colonies.

Rotavirus is a non-enveloped double-stranded RNA virus that belongs to the family Reoviridae (Yeager, Dryden, Olson, Greenberg, & Baker, 1990). The mode of transmission is believed to be fecal-oral (Chandran, Fitzwater, Zhen, & Santosham, 2010). After ingestion, this dsRNA virus enters the gastrointestinal mucosal via the epithelial cells (Greenberg & Estes, 2009). Diarrhea occurs after the destruction of the enterocyte absorption function, absorptive enzyme synthesis, and functional tight junctions between enterocytes (Ramig, 2004). Additionally, enterotoxin NSP4 (produced by rotavirus) can also increase chlorine ion secretion and gastrointestinal motility by stimulating the enteric nervous system (Ramig, 2004). All of these changes contribute to the severity of diarrhea.

We examined the prevalence of rotavirus seropositivity, rotavirus antigen shedding in stool in our breeding colony at CNPRC and we hypothesized that the rotavirus is associated with decompensated diarrhea among young rhesus macaques between 6 months and 1.5 years of age. We observed a significant association between rotavirus positivity and a recent episode

of decompensated diarrhea. While, our data did not show a significant association between the detection of rotavirus antigen in stool and symptomatic diarrhea.

METHODS

Animal Ethics Statement

Animals were maintained in accordance with the USDA Animal Welfare Act and regulations and the Guide for the Care and Use of Laboratory Animals (Animal Welfare Act as Amended. 2013. 7 USC §2131–2159, Animal Welfare Regulations. 2013. 9 CFR § 3.129. Institute for Laboratory Animal Research. 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press). The animal care and use program of the University of California, Davis is fully accredited by AAALACi, USDA-registered, and maintains a Public Health Services Assurance (National Institutes of Health. 2002. Public health service policy on humane care and use of laboratory animals. Bethesda MD: Office of Laboratory Animal Welfare). The current study was approved by the IACUC of the University of California, Davis and the research adhered to the American Society of Primatologists Principals for the Ethical Treatment of Nonhuman Primates.

Animal housing and management

All rhesus macaques (*Macaca mulatta*) were from the CNPRC outdoor breeding colony. Animals were either from the Specific-Pathogen-Free Level 1 colony (SPF1: free of simian retrovirus, simian immunodeficiency virus, simian T cell lymphotropic/leukemia virus and herpes B virus) or conventional colony (free of simian retrovirus and simian immunodeficiency virus). All the animals were socially housed in all-age and both-sex family groups. However, when animals were admitted to the hospital, they were usually individually housed for medical treatment and provided with species-appropriate environmental enrichment. Animals were fed chow ad libitum (LabDiet Monkey Diet 5047, Purina Laboratory, St. Louis, MO, USA) supplemented with fruits and vegetables biweekly and offered water ad libitum via automatic watering devices.

Study population

In this study, samples were collected from 89 macaques between 6 months and 1.5 years old (April 2017 - September 2017). Out of 89 animals, only 10 macaques with symptomatic diarrhea with secondary decompensation were identified from April to September 2017 due to the limited age range and the seasonal timing of this study. We selected animals from 6 mo to 1.5 yr of age since we focused on the age range that was experiencing the largest percentage of diarrhea. Unfortunately, the timeline for this study coordinated with the warm and dry season, not the cold and wet season when most of our diarrhea cases present. Additionally, fewer animals than normal presented this year during this time frame as well. The potential relationship between present diarrhea status with decompensation and rotavirus infection (rotavirus antigen prevalence and rotavirus antibody prevalence) was examined using a case-control study design, by taking blood and stool samples from 10 animals at time of relocation to the hospital because of identification of symptomatic diarrhea with secondary decompensation (Symp). Another 79 apparently healthy animals (Asymp) were randomly enrolled as a control group by collecting blood and stool at their

routinely scheduled health examinations. The health records for all 79 currently healthy animals were also reviewed. Of these 79 animals, 15 animals (designated as “Asymp/Symp”) were found to have a recent history of decompensated diarrhea (defined as diarrhea with secondary decompensation admitted to the hospital for more than 5 continuous days within 6 months prior to the current sample collection). The remaining 64 healthy animals (“Asymp/Asymp”) had no history of decompensated diarrhea in the prior 6 months.

Sample collection and diagnostic tests

From each animal, 3 mL of blood was drawn by venipuncture into EDTA-anticoagulated blood collection tubes. The blood samples were kept on ice and delivered directly into the refrigerator at the Primate Assay Laboratory (PAL) at CNPRC immediately after collection. The blood samples were centrifuged for 20 min at 3000 rpm within 30 minutes after collection. The plasma was collected and stored at −20°C for later diagnostic serological testing by indirect enzyme-linked immunosorbent assay (ELISA). Stool samples from the control macaques were collected by rectal swab when sedated for their routinely scheduled semi-annual physical examination. The stool samples from the diarrhea cases were collected during abdominal palpation or from their isolated hospital cage pan. Depending on the different stool quality, either 50 mg solid stool samples or 100 μL of liquid stool were expected to be collected and stored at −20°C for later tests by rotavirus antigen detection ELISA.

Serological testing for rotavirus antibody was conducted using a qualitative monkey rotavirus antibody IgG ELISA kit produced by MyBiosource (San Diego, CA). Samples and reagents were brought to room temperature (18°C-25 °C) 30 minutes prior to the test. After that, 10μL of each plasma supernatant sample was diluted with 40μL of sample diluent contained in the kit. All protocols recommended by the manufacturers were followed and all the samples were tested in duplicate. The optical density of samples was read at 450 nm with an ELISA reader at the end of test to determine the results.

Antigenic testing for rotavirus antigen was also conducted using fecal rotavirus antigen ELISA kit manufactured by Epitope Diagnostics Inc. (San Diego, CA). Formed stool (0.5–1.0 mg) diluted in 100 μl 1x Patient Sample Diluent contained in the kit before testing was centrifuged at 3000 rpm for 10 minutes and the clear supernatants were collected for antigen testing procedures. All protocols recommended by the manufacturers were followed and all the samples were tested in duplicate. The optical density of samples was read at 450 nm with an ELISA reader at the end of testing to determine the results. Unlike the antibody detection ELISA kit, the antigen detection ELISA kit used in this study is designed for humans instead of nonhuman primates specifically. Although attempts by experienced animal care staff were made to collect 50–100 mg solid or 100 ul liquid stool as suggested in the ELISA kit protocol, in some animals this amount was not available (due to the smaller size of infant macaques as compared to humans for whom the kit was designed). Since the kit instructions were to resuspend the stool sample in 1 ml of diluent and mix well before using 100 ul of the supernatant in the assay, with lesser amounts of stool the instructions were scaled down to maintain the same ratios. For example, 5 mg stool was resuspended in 100 ul diluent and all the supernatant used in the ELISA. Thus, all the stool samples were diluted to the

same ratio before testing in the ELISA. To ensure that the sensitivity of detecting rotavirus antigen was not influenced by the amount of available sample, statistical testing for the binary split of the sample amount was performed and shown to not be statistically associated with the ELISA results. There was no evidence that the amount of sample masked the true relationship between rotavirus antigen and diarrhea with secondary decompensation.

Statistical analysis

First, the two-sided Fisher's exact test was conducted to examine if there is significant relationship between rotavirus antigen results and diarrhea with secondary decompensation in the case-control study (10 Symp versus 79 Asymp). Secondly, two-sided Fisher's exact tests were conducted to examine if there is significant relationship between rotavirus antibody status and an episode of decompensated diarrhea being identified within the previous 6 months (15 Asymp/Symp versus 64 Asymp/Asymp). *P*-values less than or equal to 0.05 were considered to be significant. After that, the potential relationship between rotavirus antibody status and recent history of decompensated diarrhea was further evaluated by adjusting for other biological factors. Factors including gender, age (in days), weight (in kilograms), Specific-pathogen-free status (SPF1 or conventional status), were put into multivariate-logistic regression models along with two-way interaction terms. Akaike's Information Criterion (AIC) was used to perform stepwise selection for multivariate-logistic regression model. P-values ≤ 0.05 were considered to be significant and the final model was evaluated by Hosmer-Lemeshow test. All statistical analyses were completed in R (version 3.3.1, https://www.r-project.org).

RESULTS

Based on the ELISA results, the rotavirus antigen prevalence in stool was 36.71% (29/79) among Asymp, and 20% (2/10) among Symp (Table 1); this was not significantly different (Fisher's Exact test, p=0.48; Table 3). In contrast, if we focus on the currently healthy animal population, we observe that the rotavirus seropositivity rate was 86.67% (13/15) among Asymp/Symp and 50% (32/64) among Asymp/Asymp (Table 2, 3; Fisher's Exact P = 0.0103; odds ratio 6.3689 (95% confidence interval 1.2825– 62.6998; (Table 3).

In the next step, the differences of rotavirus antibody prevalence among currently healthy animals (Asymp/Symp versus Asymp/Asymp) was further examined with multivariate logistic regression model. Factors including age (in days), weight, gender, SPF status and rotavirus antigen test results were regarded as potential confounders and incorporated into the model selection process along with two-way interaction terms between each variable for adjustment. After performing a stepwise backward selection of the multivariable logistic regression model based on Akaike's Information Criterion (AIC), age, weight, gender and SPF status remain in the model with rotavirus antibody status. Rotavirus antibody results were still significantly related to diarrhea episodes identified within 6 months among currently healthy animals after adjusting for age, weight, gender and SPF status (P = 0.0020; Table 4, Figure1). The Hosmer-Lemeshow test indicates goodness of fit of the final model (P = 0.0886).

DISCUSSION

To our knowledge, this study provides the first prevalence survey on both rotavirus antibody and rotavirus antigen among young outdoor-housed rhesus macaques within the age range of 6 months to 1.5 years old. Our data demonstrate that rotavirus is endemic in outdoor-housed young rhesus macaque population at the CNPRC. A similar rotavirus antibody prevalence study found a higher rotavirus antibody prevalence (~ 90%) among slightly older rhesus macaques (11 months-2 years old) (Jiang, McClure, Fankhauser, Monroe, & Glass, 2004). However, the relationship between diarrhea and rotavirus infection was not examined. In another study, rotavirus was also found to be endemic in rhesus macaques colonies, however, intra-gastric administration of wild type rotavirus strain did not induce diarrhea (McNeal et al., 2005). Nevertheless, examining the association between acute diarrhea and rotavirus infection was not the main goal of that study and the sample size (10 young macaques) was not sufficient to make conclusions.

In this study, we selected animals from 6 months to 1.5 years old to exclude the possibility of maternal antibody interference (Westerman, McClure, Jiang, Almond, & Glass, 2005) and to focus on the juvenile population (< 2 years old) that suffers more frequently than other age groups from diarrhea with secondary decompensation. Decompensated diarrhea was set to be the main outcome of interest for two reasons: (1) diarrhea with secondary decompensation is the main cause of death among young macaques at the CNPRC, and (2) in an outdoor-housing environment, monitoring mild transient episodes of diarrhea is difficult. Thus, the relationship between rotavirus infection and diarrhea in general is limited in this study.

By examining with two-sided Fisher's Exact test, a significant relationship between symptomatic diarrhea with secondary decompensation and rotavirus antigen in stool was not found. In a human study, shedding rotavirus in stool samples is believed to start before the onset of diarrhea and lasts a few weeks after diarrhea (Mukhopadhya et al., 2013). In this study, the stool samples from diarrhea cases were collected after the animals were relocated to the hospital for treatment. However, we still found lower percentage of rotavirus antigen positive subjects in our Symp case group (20.00%) compared to our Asymp control group (36.71%). This may indicate that the rotavirus infection is not significantly related to decompensated diarrhea. This result may be influenced and limited by the small sample size of this case control study. There were only 10 decompensated diarrhea cases included in our study and this due to 3 main reasons; (i) we selected animals from 6 mo to 1.5 yr of age since we opted to evaluate the age range that was experiencing the peak episodes of diarrhea, (ii) a much higher occurrence of decompensated diarrhea cases is typically observed in fall and winter, which was not included in our study period (from April to September), (iii) additionally, fewer animals than normal presented this year during this time frame as well. Collected stool samples for some animal did not meet the recommended amount by the kit protocols, however the protocol does not utilize the entire amount, therefore, we scaled down and reduced the elution volume to compensate for the amount of stool. Furthermore, the statistical testing for the binary split of the sample amount (low, sufficient) and its interaction with the diarrhea status were not statistically significant (ChiSq P value = 0.26).

Upon review of the medical records, 15 animals in the healthy control group had at least one episode of decompensated diarrhea (the diarrhea was treated in hospital and lasted for five days or more) within 6 months prior to the sample collected for this study (Asymp/Symp). Additionally, all medical records were reviewed to look for any reports of diarrhea within 6 months prior to the sample collection since rotavirus antibody can remain detectable for more than 3 months after rotavirus infection (Chege et al., 2005). Based on the two-sided Fisher's Exact test, rotavirus antibody prevalence was found to be significantly related to at least one or more episodes of diarrhea with secondary decompensation within 6 months prior to our sample collection of the 79 currently healthy animals. Only currently healthy animals were included in this part of study because it takes a few weeks for rotavirus IgG antibody in the serum to be detectable after rotavirus infection (Chege et al., 2005; Westerman, Xu, Jiang, McClure, & Glass, 2005) and other animals that were hospitalized for reasons other than diarrhea were not sampled in this study. Hence, including animals with decompensated diarrhea can lead to a selection bias of the analysis. After discovering the significant relationship between rotavirus antibody and episodes of decompensated diarrhea within the past 6 months prior to sample collection, we used a multivariate logistic regression model to examine this relationship by adjusting other biological factors. By adjusting for age, weight, gender and SPF status, rotavirus antibody results are still significantly related to decompensated diarrhea within 6 months prior to sample collection. In the multivariate logistic regression model, wider confidence intervals were observed with two explanatory variables (rotavirus antibody results and SPF status). This sign of instability of the model can be caused by the imbalanced distribution and the limited sample size of study subjects (Figure 1).

This is the first study that indicates the possible relationship between diarrhea and rotavirus infection among juvenile macaques. Although, there was no statistically significant association between the rotavirus antigen results and diarrhea with secondary decompensation within past 6 months, we found a statistically significant rotavirus antibody status association with diarrhea with secondary decompensation within past 6 months which reject the null hypothesis, and supports the alternative hypothesis that the rotavirus is associated with decompensated diarrhea among young rhesus macaques between 6 months and 1.5 years of age.

We should note that the relationship between decompensated diarrhea during infant period (< 6 months old) and rotavirus infection cannot be accessed in this study because we did not include animals less than 6 months of age to avoid the potential confounding effect of maternal antibodies. Although, we found that rotavirus is associated with the diarrhea with secondary decompensation, the causality of the agent cannot be proven in this study. And we recommend conducting a randomized controlled trial to test the causal relationship of the agent and control the other causes of infant diarrhea (such as other viruses, parasites, bacteria, diet or metabolic diseases).

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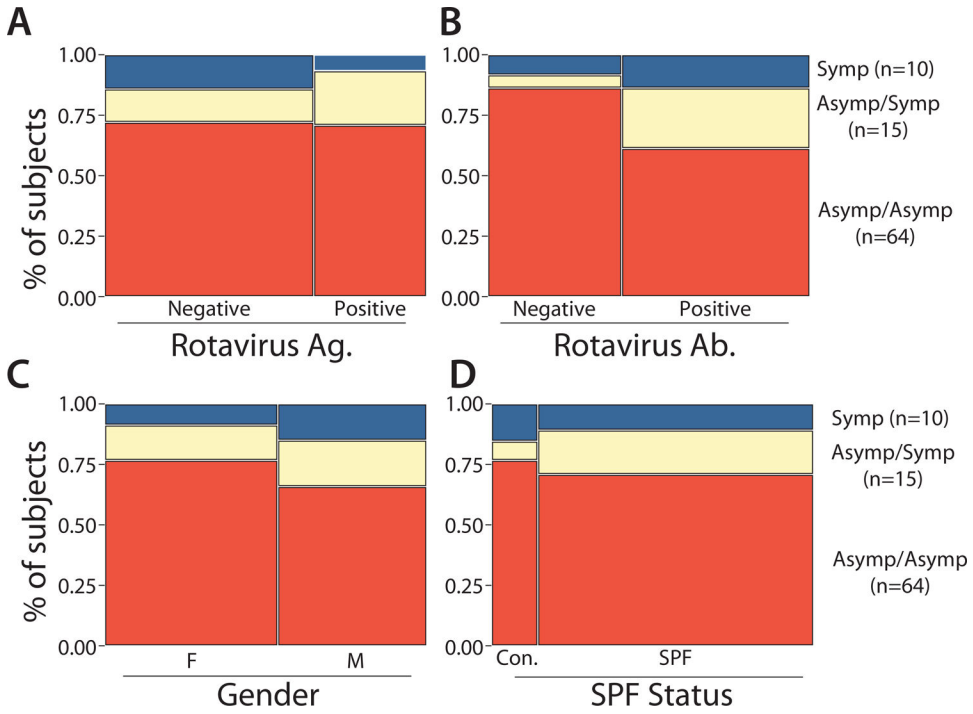

Figure 1. Mosaic plots represent the proportion of the observations in classes for one variable across classes of the second variable.

Plots represent the symptomatic diarrhea with secondary decompensation (Symp, n=10), healthy animals at the time sampling with a recent (within 6 months) history of decompensated diarrhea (Asymp/Symp, n=15) and healthy animals at the time of sampling with no history of decompensated diarrhea in the prior 6 months (Asymp/Asymp, n=64) and their **(A)** rotavirus Ag., **(B)** rotavirus Ab., **(C)** Gener, and **(D)** their SPF status.

Table 1.

Rotavirus antigen prevalence among Symp and Asymp.

	Symp	Asymp
	Number (%)	Number (%)
Whole sample population	10	79
Rotavirus antigen		
Positive	2 (20)	29 (36.71)
Negative	8 (80)	50 (63.29)

Rotavirus antigen prevalence among decompensated diarrhea cases (Symp, n= 10) and apparently healthy group (Asymp, n= 79).

Table 2.

Rotavirus antibody prevalence among Asymp/Symp and Asymp/Asymp

	Asymp/Symp	Asymp/Asymp
	Number (%)	**Number (%)**
Whole sample population	15	64
Rotavirus antibody		
Positive	13 (86.67)	32 (50)
Negative	2 (13.33)	32 (50)

Rotavirus antibody prevalence among animals that are apparently healthy but with decompensated diarrhea history within past 6 months (Asymp/Symp, n= 15) and animals that are apparently healthy with no decompensated diarrhea history (Asymp/Asymp, n= 64).

Table 3.

Fisher's exact test of rotavirus antigen prevalence or serological status results between study groups

	Odds ratio (95% CI)	P-value
†Rotavirus antigen (n=89)		
Symp (n=10)	0.4347 (0.0422–2.3876)	0.4838
Asymp (n=79)	1.00	
‡Rotavirus antibody status (n=79)		
Asymp/Symp (n=15)	6.3689 (1.2825–62.6998)	0.0103*
Asymp/Asymp (n=64)	1.00	

†Comparison for rotavirus antigen prevalence between decompensated diarrhea cases (Symp) and apparently healthy animals (Asymp);

‡Comparison of rotavirus antibody status between apparently healthy animals with decompensated diarrhea history within past 6 months (Asymp/Symp) and animals that are apparently healthy with no decompensated diarrhea history (Asymp/Asymp).

*indicates significance

Table 4.

Multivariable logistic regression model

	Odds ratio (95% CI)	P-value
Age (in days)	1.0154 (1.0018–1.0293)	0.0165*
Weight (in kilograms)	0.0063 (0.0003–0.1646)	0.0001*
Sex		0.0295*
Male	5.4812 (1.0581–28.3938)	
Female (reference group)	1.00	
SPF status		0.0167*
SPF-1	12.2219 (1.0755–138.8936)	
Conventional (reference group)	1.00	
Rotavirus antibody status		0.0020*
Positive	11.6743 (1.8212–74.8354)	
Negative (reference group)	1.00	

Rotavirus antibody results remain significantly related to the outcome of multivariable logistic regression model (decompensated diarrhea within past 6 months) after adjusting for other biological factors.

* indicates significance