Original Article

Identification of Rotavirus Strains Causing Diarrhoea in Children under Five Years of Age in Yogyakarta, Indonesia

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Abstract

Background: **Rotavirus is an important cause of severe diarrhoea in children. The aims of this study were to identify the rotavirus strains that cause diarrhoea in children in Yogyakarta and to determine the association between rotavirus positivity and its clinical manifestations.**

Methods: **Clinical data and stool samples were collected from children hospitalised at Kodya Yogyakarta Hospital, Indonesia. Rotavirus was detected in stool samples using an enzyme immunoassay (EIA), which was followed by genotyping using reverse transcriptase polymerase chain reaction (RT-PCR). Electropherotyping was performed for the rotavirus-positive samples.**

Results: **In total, 104 cases were included in the study, 57 (54.8%) of which were rotavirus-positive. Based on a multiple logistic regression analysis, age group, vomiting and stool mucous were associated with rotavirus positivity. Most of the 56 samples subjected to genotyping were classified as G1 (80.36%) and P[8] (69.64%) genotypes. The genotype combination G1P[8] was identified as the most prevalent strain (66.07%). Of the 19 samples subjected to electropherotyping, 17 G1 isolates and 1 G3 isolate had long patterns, and 1 G1 isolate had a short pattern.**

Conclusion: **G1P[8] was the most dominant strain of rotavirus causing diarrhoea in children in Yogyakarta. Age group, vomiting and stool mucous were associated with rotavirus positivity.**

Keywords: *rotavirus, diarrhoea, G-type, P-type, children*

Introduction

Rotavirus is an RNA virus that mainly infects the gastrointestinal tract. It is the most prevalent agent causing severe diarrhoea among infants and young children in both developed and developing countries, including Indonesia (1–3). It was recently estimated that rotavirus is responsible for 7,200,000–11,500,000 severe diarrhoea episodes and 133,000–284,000 deaths, most of which occur in developing countries (2). The incidence might be lower in countries that have introduced rotavirus vaccination (2).

Since rotavirus diarrhoea is prevalent among children in developed and developing countries, it is unlikely that improvements in sanitation and hygiene will be able to lower the incidence of the disease. Therefore, one of the best strategies for decreasing the global burden of disease is development and implementation of effective vaccines (1). The current strategy for developing rotavirus vaccines involves oral administration of live attenuated viruses that protection is expected to be similar to natural rotavirus infection (4, 5).

Rotavirus has three essential antigenic specificities, determined by the viral proteins VP6, VP7 and VP4. Based on the VP6 protein, rotavirus is classified into seven groups (A to G), yet only groups A, B and C infect humans. VP7, a glycoprotein, determines G-genotype, while VP4, which is protease-sensitive, determines P-genotype. VP7 and VP4 form the outer layer of rotavirus and elicit neutralising antibody responses. Therefore, they are implicated in the dual classification of rotavirus group A as G- and P-type (6).

The effectiveness of rotavirus vaccine can be enhanced by identification of the patterns of circulating virus serotypes and the role of these serotypes in causing clinical illness (7). During vaccine implementation, strain surveillance is necessary to monitor antibody-escape variants and changes in the circulating rotavirus strains among humans (8).

Two rotavirus vaccines, Rotarix and RotaTeq, have been developed by GSK and Merck, respectively. RotaTeq is a live attenuated pentavalent vaccine containing G1, G2, G3, G4 and P[8] rotavirus derived from reassortants of human and bovine strains. Rotarix is a live attenuated monovalent vaccine derived from the most common human rotavirus strain, G1P[8]. These vaccines have been proven to be highly effective for the global prevention of severe diarrhoea (9, 10). Before implementing the rotavirus vaccination programme in Yogyakarta, it is crucial to perform a study to ensure that the vaccines used in the programme match with the most prevalent and clinically relevant rotavirus strain in Yogyakarta.

The aims of this study were to identify the rotavirus strains currently circulating in Yogyakarta and to determine the association between rotavirus positivity and the illness's clinical manifestation. This study provides accurate information about the rotavirus strains circulating in Yogyakarta before and after the implementation of the vaccination programme.

Materials and Methods

Study population and case definition

From February to August 2009, we enrolled 104 children under five years of age that were hospitalised at Kodya Yogyakarta General Hospital, Indonesia due to acute diarrhoea. We defined acute diarrhoea as 3 or more instances of loose stool within 24 hours for a duration of fewer than 2 weeks (11). We excluded patients whose faecal samples could not be obtained within 48 hours of admission to the hospital.

Clinical data collection

The patients' medical histories, including previous occurrences of blood and mucous in stool, fever, vomiting, dehydration and diarrhoea, were collected.

Sample collection and storage

Stool samples were collected within the first 48 hours after admission according to WHO protocol (12). Stool specimens were stored at 4 °C – 8 °C before they were transported to the Department of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, Indonesia. The specimens were then aliquoted into several tubes and stored at -20 °C.

Detection of rotavirus in stool samples

All stool samples were examined for the presence of group A rotavirus by enzyme immunoassay (EIA) using the IDEIA™ Rotavirus (DakoCytomation) kit according to the manufacturer's instructions. Other possible causes of diarrhoea were not examined.

RT-PCR genotyping

Rotavirus RNA was extracted from rotavirus-positive faecal specimens using Trizol (Invitrogen) according to the manufacturer's instructions. Rotavirus RNA was analysed to determine the VP7 (G-type) and VP4 (P-type) genotypes using reverse transcriptase polymerase chain reaction (RT-PCR) as previously described (13–15). For G typing, the primers 9con1L (13) and VP7R (15) were used during the first round of RT-PCR (20 cycles) to amplify the 905-bp of the VP7 gene segment. Then, 9con1L was used in the second round of PCR (20 cycles) with the type-specific primers 9T1 (G1), 9T-2 (G2), 9T-3P (G3), 9T4 (G4) and 9T-9B (G9) (Table 1) (13). For P-typing, the primers con2 and con3 were used in the first-round of RT-PCR (20 cycles) to amplify the 877-

Table 1. Primers used for VP7 genotyping of rotavirus strains

Primer		Sequence (5'-3')	Position	PCR product (bp)	References
First amplification of G-typing as described by Das et al. (13) and Gomara et al. (15)					
	9con1L	TAG CTC CTT TTA ATG TAT GGT AT	37–59	895	Das BK et al. (13)
	VP7R	AAC TTG CCA CCA TTT TTT CC	914–932		Gomara MI et al. (15)
First amplification of G-typing as described by Gomara et al. (15)					
	VP7F	ATG TAT GGT ATT GAA TAT ACC AC	51–71	881	Gomara MI et al. (15)
	VP7R	AAC TTG CCA CCA TTT TTT CC	914–932		Gomara MI et al. (15)
Second amplification of G-typing as described by Das et al. (13)					
G1	9T-1	TCT TGT CAA AGC AAA TAA TG	176–195	158	Das BK et al. (13)
G2	9T-2	GTT AGA AAT GAT TCT CCA CT	262–281	244	Das BK et al. (13)
G3	9T-3P	GTC CAG TTG CAG TGT AGC	484–501	464	Das BK et al. (13)
G4	9T-4	GGG TCG ATG GAA AAT TCT	423–440	403	Das BK et al. (13)
G9	9T-9B	TAT AAA GTC CAT TGC AC	131–147	110	Das BK et al. (13)
Second amplification of G-typing as described by Gouvea et al. (16)					
G1	aBT1	CAA GTA CTC AAA TCA ATG ATG G	314–335	618	Gouvea et al. (16)
G2	aCT2	CAA TGA TAT TAA CAC ATT TTC TGT G	411–435	521	Gouvea et al. (16)
G3	G3	ACG AAC TCA ACA CGA GAG G	250–259	682	Gouvea et al. (16)
G4	aDT4	CGT TTC TGG TGA GGA GTT G	480–498	452	Gouvea et al. (16)
G8	aAT8	GTC ACA CCA TTT GTA AAT TCG	178–198	754	Gouvea et al. (16)
G9	aFT9	CTT GAT GTG ACT AYA AAT AC	757–776	179	Gouvea et al. (16)

bp fragment of the VP4 gene segment (14). Con3 was used in the second round of PCR (30 cycles) with the type-specific primers 1T-1D (P[8]), 2T-1 (P[4]), 3T-1(P[6]), 4T-1 (P[9]) and 5T-1 (P[10]) (Table 2) (14).

Samples that failed to yield a detectable PCR product from secondary amplification of the primer-specific PCR typing were re-typed with other primers using the methods described by Gouvea et al. (16) and Simmond et al. (8). For G typing, the consensus primers VP7F and VP7R were used in the first round of RT-PCR (30 cycles) to generate the 881-bp of the VP7 gene segment (15). VP7F was used in the second round of PCR (30 cycles) with the type-specific primers aBT1 (G1), aCT-2 (G2), G3 (G3), aDT4 (G4) and G9 (G9) (Table 1) (16). For P-typing, the consensus primers VP4F and VP4R were used in the first round of RT-PCR (30 cycles) to generate a 663-bp fragment of the VP4 gene segment (8). VP4F was used in the second round of PCR (40 cycles) with the type-specific primers 1T-1D (P[8]), 2T-1 (P[4]), 3T-1(P[6]), 4T-1 (P[9]) and 5T-1 (P[10]) (Table 2) (14). All PCR products were separated in 2% agarose gel and visualised under UV light after staining with ethidium bromide.

Electropherotyping

Rotavirus RNA was extracted using Minikit (Qiagen) according to the manufacturer's instructions. RNA was subjected to polyacrylamide gel electrophoresis to separate the 11 segments of dsRNA, which were then visualised by silver staining.

Statistical analyses

The variables were described using frequencies and percentages. Chi-square tests and logistic regression analyses were used to determine the association between rotavirus infections and clinical manifestations using STATA 13. The variables that had *P*-values of less than 0.25 according to the chi-square tests were entered into a logistic regression analysis model. Age group and all the clinical symptoms analysed (vomiting, stool mucous, bloody stool and fever) had *P*-values of less than 0.25 (Table 3). Bloody

Table 2. Primers used for VP4 genotyping of rotavirus strains

Primer	Sequence (5'-3')	Position	PCR product (bp)	References
First amplification of P-typing as described by Gentsch et al. (14)				
Con-3	TGG CTT CGC TCA TTT ATA GAC A	11–32	876	Gentsch et al. (14)
Con-2	ATT TCG GAC CAT TTA TAA CC	868–887		Gentsch et al. (14)
First amplification of P-typing as described by Simmonds et al. (8)				
VP4F	TAT GCT CCA GTN AAT TGG	132–149	663	Simmonds et al. (8)
VP4R	ATT GCA TTT CTT TCC ATA ATG	775–795		Simmonds et al. (8)
Second amplification of P-typing as described by Gentsch et al. (14)				
P[4]	2T-1	CTA TTG TTA GAG GTT AGA GTC	474–494	483	Gentsch et al. (14)
P[6]	3T-1	TGT TGA TTA GTT GGA TTC AA	259–278	267	Gentsch et al. (14)
P[8]	1T-1D	TCT ACT TGG ATA ACG TGC	339– 356	345	Gentsch et al. (14)
P[9]	4T-1	TGA GAC ATG CAA TTG GAC	385–402	391	Gentsch et al. (14)
P[10]	5T-1	ATC ATA GTT AGT AGT CGG	575–594	583	Gentsch et al. (14)

stool was omitted because the chi-square test revealed cells with a count of zero. Therefore, age group, vomiting, stool mucous and fever were examined in the multiple logistic regression analysis. The fit of the model was checked using the Hosmer-Lemeshow test. The results were presented as crude and adjusted odds ratios with a 95% confidence interval. *P*-values of less than 0.05 were considered statistically significant.

Ethical approval

The research protocol was approved by the Ethical Committee of the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. Informed consent was provided by the parents or guardians of each child before the children were enrolled in the study.

Results

From February to August 2009, 104 children under five years of age who were hospitalised at Kodya Yogyakarta General Hospital, Indonesia due to acute diarrhoea were enrolled in the study. There were 70 (67.3%) male and 34 (32.7%) female patients (Table 3). Of the 104 samples collected, 57 (54.8%) were positive for rotavirus, as determined by EIA.

Rotavirus diarrhoea particularly affects young children; 70.2% of the cases involved children under two years of age. Most cases involved children aged 12–23 months, with the next largest group comprised of children aged 24–59 months (Table 3). Additionally, the chi-square tests revealed significant differences in vomiting, stool mucous and bloody stool between rotavirus-positive and rotavirus-negative patients (Table 3).

We present the final model of the multiple logistic regression analysis in Table 4. There are four variables associated with rotavirus positivity: age group, including the 0–11-month-old group (adjusted OR = 0.31; CI 95% = 0.10 to 0.93; P = 0.04) and 12–23-month-old group (adjusted OR = 3.46; CI 95% 1.03 to 11.60; P = 0.04), stool mucous (adjusted OR = 0.24; CI 95% = 0.07 to 0.79; P = 0.02), and vomiting (adjusted OR = 3.97; CI 95% = 1.32 to 11.97; P = 0.01). In our study, only one patient had mild dehydration, and all the others (n = 103) had no dehydration.

Of the 57 rotavirus-positive samples, 56 faecal specimens were characterised as G or P genotypes. G1 was the most prevalent genotype (80.36%), followed by G2 (16.07%) and G3 (3.57%) (Table 5). Regarding P-typing, they were classified as P[8] (69.64%), P[4] (17.86%), P[6] (7.14%) or untypeable (5.36%) (Table 5). The identified G- and P-type combinations were G1P[8] (66.07%), G2P[4] (16.07%), G1P[6] (7.14%), G1P[untypeable] (5.36%), G3P[8] (3.57%) and G1P[4] (1.79%) (Table 5).

A number of rotavirus-positive stool samples did not have sufficient volume for polyacrylamide gel electrophoresis (PAGE) analysis. Only 24 samples were subjected to PAGE analysis, and 19 isolates had good results. Eighteen samples were identified as G1, and

Table 3. Characteristics and clinical symptoms of children under five years of age hospitalised at Kodya Yogyakarta General Hospital (n = 104)

Variable		n (%) of patients with rotavirus-positive diarrhoea (n = 57)	n (%) of patients with rotavirus-negative diarrhoea (n = 47)	n	X^2-Statistic[a] (df)	P-value[a]
Gender	Male	41 (71.9)	29 (61.7)	70	1.22 (1)	0.27
	Female	16 (28.1)	18 (38.3)	34		
Age groups	0–11	11 (19.3)	25 (53.2)	36	4.57 (1)	0.03*
	12–23	29 (50.9)	9 (19.1)	38	2.96 (1)	0.08
	24–59	17 (29.8)	13 (27.7)	30		
Clinical manifestation	Vomiting	46 (80.7)	27 (57.4)	73	6.66(1)	0.01*
	Stool mucous	6 (10.5)	17 (36.2)	23	9.83 (1)	0.00*
	Bloody stool	0 (0)	11 (23.4)	11	14.92 (1)	0.00*
	Fever	18 (31.6)	23 (48.9)	41	3.25 (1)	0.07

[a] chi-square test for independence

Table 4. Association between age groups, clinical symptoms and rotavirus positivity revealed by multiple logistic regression

Variable	b	Adjusted OR (95% CI)	P-value[a]
Age group: 0–11 months vs. 24–59 months[b]	-1.17	0.31 (0.10–0.93)	0.04
Age group: 12–23 months vs. 24–59 months[b]	1.24	3.46 (1.03–11.60)	0.04
Vomiting	1.38	3.97 (1.32–11.97)	0.01
Stool mucous	-1.41	0.24 (0.07–0.79)	0.02

OR = odds ratio. [a] Likelihood ratio test; [b] reference category.

one sample was identified as G3. All of them showed the migration pattern of the genome of rotavirus group A: 4-2-3-2. The results of electropherotyping showed that 17 of 18 G1 isolates and 1 G3 isolate had long patterns, while 1 G1 isolate had a short pattern (Figure 1).

Discussion

This hospital-based study described a high incidence of rotavirus infection in Yogyakarta, Indonesia. Rotavirus infection was identified in 54.8% of 104 children hospitalised with acute diarrhoea. Compared to previous hospital-based studies performed in Indonesia in the late 1970s and 1990s, the incidence rate was similarly high. Bishop et al. (17) reported rotavirus infection in 38% of children with severe acute diarrhoea in Yogyakarta. Subekti et al. (18), who conducted a study in Jakarta from 1997–1999, showed that 170 of 236 (72%) children with diarrhoea were infected with rotavirus. Putnam et al. (19), who conducted a study in Medan, Makassar, Jakarta, Yogyakarta, Surabaya, Bali and Nusa Tenggara, found that 748 of 1,660 (45.1%) children under five years of age suffered from diarrhoea due to rotavirus. Moreover, Soenarto et al. (20), who conducted a study of children under five years of age in Yogyakarta, Jakarta, Bandung, Mataram, Denpasar and Palembang in 2006, showed that 60% of the 2,240 children studied suffered from diarrhoea due to rotavirus. These findings support the need for continuous surveillance of the disease in various locations.

Our study found a high incidence of rotavirus infection in Yogyakarta, Indonesia, which is comparable to the findings of similar studies in surrounding countries, such as Malaysia (38%) (21), Thailand (43%) (22), Cambodia (56%) (23) and Myanmar (57%) (24). Our study provides evidence that rotavirus infection gives a substantial burden to the

Table 5. Rotavirus genotype distribution in Kodya Yogyakarta Hospital

Rotavirus strains	Total (%) (*n*=56)
G genotype	
G1	45 (80.36)
G2	9 (16.07)
G3	2 (3.57)
P genotype	
P[8]	39 (69.64)
P[4]	10 (17.86)
P[6]	4 (7.14)
P[untypeable]	3 (5.36)
G- and P-type combinations	
G1P[8]	37 (66.07)
G2P[4]	9 (16.07)
G1P[6]	4 (7.14)
G1P[untypeable]	3 (5.36)
G3P[8]	2 (3.57)
G1P[4]	1 (1.79)

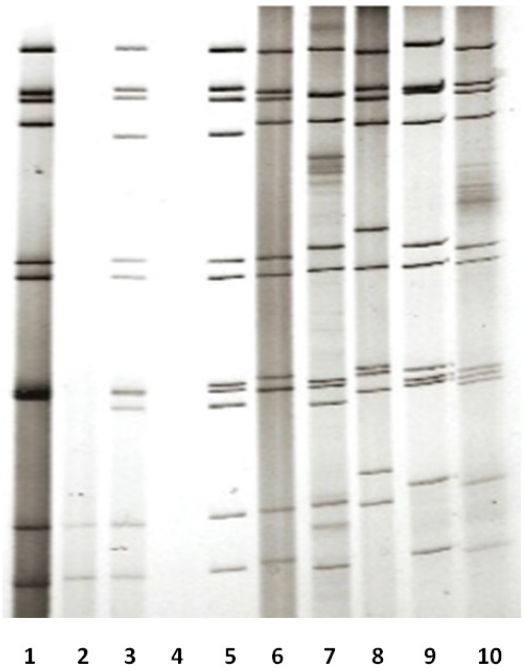

Figure 1. Electropherotype profile of rotavirus RNA genome. Lanes 1–5: sample RW13 (G3P[8]) - long pattern; sample RW22 - RNA insufficient; sample RW23 (G1P[8]) - long pattern; sample RW26 - no RNA; sample RW30 (G1P[8]) - long pattern. Lanes 6–10 are control strains: RV3 (G3P[6]) - long pattern; RV4 (G1P[8]) - long pattern; RV5 (G2P[4]) - short pattern; F45 (G9P[8]) - long pattern; ST3 (G4P[6]) - long pattern.

diarrhoeal diseases and it is critical to develop an effective approach to preventing infection.

Rotavirus infection in children clinically manifests with several symptoms, such as fever, vomiting and watery, bloodless diarrhoea (25, 26). These clinical manifestations are more severe than those of other causes of viral gastroenteritis (27). In our study, vomiting was associated with rotavirus positivity (Table 4), which aligns with the findings of other studies (20, 28). Those studies also reported that rotavirus-positive patients were less likely to have bloody diarrhoea (20, 28), in agreement with our study.

Severe complications of rotavirus infection, such as bloody diarrhoea associated with necrotising enterocolitis (NEC) and bowel perforation, have been described (25, 26, 29). Even though they have not yet been fully elucidated, several factors were associated with the pathogenesis of the disease (30). However, an enterotoxin protein, NSP4, and the ability of rotavirus to trigger the apoptosis of intestinal cells might be the factors that most affect the manifestation of the disease (31).

Our study demonstrated that the percentage of children aged 0–11 months who suffered from rotavirus diarrhoea was lower than that of children aged 12–23 and 24–59 months (Table 3). This phenomenon is probably due to the presence of anti-rotavirus immunoglobulin G (IgG) and IgA antibodies in breast milk (32). In their study of children in Yogyakarta, Chan et al. (33) found a high concentration of anti-rotavirus IgG and IgA in colostrum as well as in breast milk. Moreover, Clemens et al. (34) found that exclusive breastfeeding confers better protection against severe rotavirus infection than partial or no breastfeeding. However, further study is required to fully address this issue in our patient population.

The genetic diversity of circulating rotavirus strains may have a crucial role in the initiation of the vaccination programme and its clinical evaluation. Some studies have been conducted to identify the rotavirus strains circulating in Indonesia. Using samples collected in Yogyakarta from 1978–1979, Bishop et al. (17) identified G1 (2%); G2 (9%), G3 (53%) and G4 (36%) strains.

However, Soenarto et al. (20) reported that the G1 and G9 genotypes were the most prevalent, accounting for 73 (30%) and 69 (29%) of the 240 samples, respectively, followed by G2, which accounted for 34 samples (14%). Putnam et al. (19) reported that the rotavirus strains causing diarrhoea in Indonesia were P[4] (16.2%), P[6] (11.3%), P[8] (24.7%), P[9] (0.7%), P[10] (1.3%), P[11] (0.5%), mixed infection (3.6%) and untypeable (1.6%). However, Soenarto et al. (20) found that the P-type rotavirus strains causing diarrhoea were distributed differently: P[4] (16.4%), P[6] (55.6%), P[8] (17.5%), mixed infection (9%) and untypeable (1.6%).

We found that G1P[8] was the most dominant strain of rotavirus causing diarrhoea in children under five years of age hospitalised at Kodya Yogyakarta Hospital. G1P[8] was also the predominant strain identified in Malaysia in a 1996 study (35). However, G9P[8] genotypes became the most common strains in Malaysia from 2001–2003 and in 2007 (21, 35).

In our study, G2P[4], G1P[6], G3P[8] and G1P[4] were also common causative agents. Since the majority of rotavirus strains circulating in Yogyakarta are similar to those circulating globally, we suggest that the current rotavirus vaccines can effectively reduce the prevalence of rotavirus diarrhoea in children under five years of age in Yogyakarta.

Rotavirus has several mechanisms to generate genetic diversity. One is accumulation of point mutation (genetic drift), which is due to the error-prone nature of RNA-dependent RNA polymerase (RdRp) enzyme (36). Such mutations could be introduced at the primer binding sites and result in typing failure (8, 15). Another important mechanism of rotavirus evolution is genetic shift due to reassortment of the segmented RNA genome. This event could lead to a new combination of G and P types (36) and introduce novel genotypes through zoonotic transmission (37). Both mechanisms may explain our finding that the P-type of about 5% of samples could not be identified. The rapid evolution of rotavirus may increase the number of strains that cannot be genotyped with currently available assays. Therefore, our study underscores the importance of regularly updating typing methods to successfully detect the currently circulating rotavirus strains.

Continuous monitoring is important for identifying circulating rotavirus strains before and after the introduction of the vaccine. It will provide information about the impact of the vaccine on genotype distribution and the possible emergence of less common rotavirus strains circulating in certain geographical areas. It is also crucial to assess the effectiveness of the vaccination programme against rotavirus diarrhoea.

According to the migration of its 11 RNA gene segments, rotavirus can also be classified as either long or short RNA electropherotype using polyacrylamide gels. The short electropherotype occurs as a result of partial duplication in gene segment 11. This gene runs more slowly than gene segment 10; because of its standard size, gene segment 11 in long-electropherotype strains migrates faster than segment 10 (38). In this study, we found that most G1 and G3 isolates had a long electropherotype. Long electropherotypes usually belong to the Wa genogroup, whereas short electropherotypes typically belong to the DS-1 genogroup (36).

Conclusion

We found that rotavirus caused acute diarrhoea in 54.8% of children under five years of aged hospitalised at Kodya Yogyakarta General Hospital. The majority (66.07%) was G1P[8], the most prevalent strain identified globally. Age group, vomiting and stool mucous were associated with rotavirus positivity.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

Conception and design: HN, ATA
Analysis and interpretation of the data: HN, MSH
Drafting of the article: HN, MSH
Critical revision of the article for important intellectual content: QP
Final approval of the article: QP, ATA
Provision of study materials or patients: SA
Obtaining of funding: ATA
Administrative, technical, or logistic supports: IBNPD
Collection and assembly of data: HN, MSH, IBNPD

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