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Monoplex Nested and Semi-nested Reverse Transcription Polymerase Chain Reaction for G and P Genotyping of Human Rotavirus Group A in Clinical Specimens and Environmental Samples

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ABSTRACT

Introduction: Human rotavirus group A genotyping is important to monitor changes in rotavirus genotypes distribution. Although multiplex reverse transcription polymerase chain reaction (RT-PCR) assays are widely used for genotyping of rotavirus strains, they may be less sensitive than monoplex RT-PCR assays

Objective: To use monoplex nested and semi-nested RT-PCR for re-examination of human rotavirus group A common G and P genotypes respectively in two hundred and fifty stool specimens and twenty-four raw sewage samples which were examined in a previous study for human rotavirus group A common G and P genotypes using multiplex nested and semi-nested RT-PCR respectively.

Results: Increasing the number of typeable G and P specimens were observed in this study in comparison to the previous study. Rotavirus common G genotypes were detected in 57.60% (144/250) of the positive clinical specimens. The most frequent G genotype was G1 (29.60%), followed by G3 (23.20%). Both of them were with a significantly higher prevalence than there prevalence in the previous study. Genotypes G2 and G4 which were absent in the previous study had appeared in this study. Genotype G2 had been detected in 2.80% of the specimens, while genotype G4 had been detected in 2.00% of the specimens. In the raw sewage samples, genotype G1 was detected in a higher percentage (37.50%) than the previous study. Genotypes G2 and G4 were detected in 4.17% for each of them, while they were absent in the previous study. Rotavirus common P genotypes were detected in 92.80% (232/250) of the positive clinical specimen. The most frequent P genotype was P[4] (41.60%) followed by P[8] (24.00%), P[6] (20.40%). All of them were with significantly higher prevalence than their prevalence in the previous study. In the raw sewage samples, genotype P[4] was detected in higher percentage (41.67%) than the previous study.

Conclusion: This study has shown that nested and semi-nested monoplex RT-PCR is more sensitive than nested and semi-nested multiplex RT-PCR in genotyping of human rotaviruses.

Key Words: Egyptian infants, Human Rotavirus Group A, Genotyping, Monoplex, RT-PCR, Raw sewage

INTRODUCTION

Rotaviruses belong to the genus *Rotavirus* within the *Reoviridae* family and its genome consists of eleven dsRNA segments. These viruses are distinct in that their segmented genome undergoes reassortment during replication.[1] According to the classification system based on the gene sequence of VP6 an inner capsid protein, rotaviruses are currently categorized into nine groups (A-I).[2] Rotavirus group A is a major etiologic agent of acute gastroenteritis in children under 5 years of age and is associated annually with approximately 215,000 deaths worldwide.[3] Rotaviruses group A are classified based on the molecular characterization of the two outer capsid proteins, VP7 (glycoprotein) and VP4 (protease-sensitive) into different G and P genotypes respectively.[4] At least 36 G genotypes and 51 P genotypes of group A rotavirus had been reported to cause

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infection in humans and animals.[5] The most prevalent rotavirus genotypes detected around the world include G1, G2, G3, and G4, in association with the most prevalent human P-genotypes P[4], P[6], and P[8].[6-7]

In a previous study in Egypt, the frequencies of P genotypes were as follows: P[8], 53.33%; P[6], 30.00%; and P[4], 16.67%, the most frequent G type was G1 (69.60%), followed by G3 (13.00%), G4 (8.70%), and G9 (8.70%).[8] Also, previous studies reported that rotavirus group A was the most frequent RNA enteric viruses in Egyptian clinical specimens and aquatic environment and was the most resistant one to sewage and water treatment processes.[9-17] Rotavirus group A is more frequent in Egyptian clinical specimens and environmental samples than rotavirus group C.[18]

Currently, two live attenuated oral rotavirus vaccines, Rotarix® (derived from a single strain of human rotavirus G1P[8]) and Rotateq® (containing five reassortant bovine-human rotaviruses, G1-4P[5] and G6P[8]) have been licensed and used extensively in >100 countries worldwide since 2006.[19] However, these vaccines are live attenuated oral vaccines that can replicate in the intestine and had been reported to shed in faeces after vaccination.[20-21] Also, reassortant vaccine strains derived from Rotarix® and Rotateq® had been reported to cause acute gastroenteritis in vaccinated and unvaccinated children.[22-28]

Thus, rotavirus genotyping is important to monitor changes in rotavirus genotypes distribution and to notice the emergence of novel rotavirus strains that not covered serotypically by the currently available rotavirus vaccines. Molecular techniques based on multiplex RT-PCR assays are widely used for genotyping of rotavirus strains. In particular, the VP7 primer set reported by Gouvea and colleagues is still widely used worldwide as the recommended WHO primer set in regional and national reference RVA surveillance laboratories.[29] Although multiplex RT-PCR assays offer several advantages, including reduced labor and reagent costs and potentially faster detection, it may be less sensitive than monoplex RT-PCR assays in the detection of pathogens. In our previous study, we examined the common G (G1-G4) and common P (P[8], P[4], and P[6]) genotypes using multiplex nested and semi-nested RT-PCR respectively and a large number of G-untypeable cases was observed.[31] Thus, the objective of this study was to re-examine the two hundred and fifty stool specimens and the twenty-four raw sewage samples which were previously positive for rotavirus VP6 for common G and P genotypes using monoplex nested and semi-nested RT-PCR respectively and to compare them with our previous results reported by El-Senousy and co-workers which multiplex nested and semi-nested RT-PCR to investigate common G and P genotypes were used.[31]

MATERIALS AND METHODS

Clinical Specimens and Raw Sewage Samples

Two hundred and fifty stool specimens which were previously positive for rotavirus group A VP6 were re-examined for G and P genotypes using monoplex nested and semi-nested RT-PCR respectively.[31] These specimens were collected from children ≤2 years of age from Abo El-Reech hospital in Greater Cairo, Egypt from October 2015 to September 2017. Also, 24 raw sewage samples which were previously positive for rotavirus VP6 were re-examined for G and P genotypes using monoplex nested and semi-nested RT-PCR respectively. These samples were collected from inlets of El-Gabal El-Asfar and Zenin wastewater treatment plants (WWTPs) in autumn and winter months from October 2015 to March 2016 and from October 2016 to March 2017.

Monoplex Nested RT-PCR for Human Rotavirus Group A G Genotyping

Human rotavirus group A G genotyping was performed according to Gouvea et al. with some modifications in the second nested RT-PCR using monoplex primers instead of multiplex primers.[29] The first round RT-PCR amplified the whole gene segment 9 (coding for VP7) (1062) using primers Beg9 5′-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3′ and End9 5′- GGTCACATCATACAATTCTAATCTAAG-3′. The second-round PCR was a monoplex nested PCR and included the primer pair RVG9 5′- GGTCACATCATACAATTCT-3′ and a single G-type specific primer aBT1 (G1 specific) 5′-CAAGTACTCAAATCAATGATGG-3′, or aCT2 (G2 specific) 5′-CAATGATATTAACACATTTTCTGTG-3′, or aET3 (G3 specific) 5′-CGTTTGAAGAAGTTGCAACAG-3′, or aDT4 (G4 specific) 5′-CGTTTCTGGTGAGGAGTTG-3′with a predicted product size of 749 bp, 652 bp, 374 bp, and 583 bp respectively.

The PCR mixture for the first round consists of 5 µl of the PCR buffer (Roche), 0.2 mM of each dNTP's 1 U of Expand PCR enzyme (Roche) and 1 µM concentration of each primer. The first-round PCR was performed by adding 5 µl of cDNA [primed with 1 µM of both Beg 9 and End 9, 0.2 mM of dNTP's and 3 U of RT enzyme (Roche); RT was done at 50°C for 1h] to 45 µl of PCR mixture. After denaturation at 95°C for 9 min, 40 PCR cycles each consisting of 94°C for 1 min, 47°C for 2 min, and 72°C for 5 min were performed, followed by an extension at 72°C for 10 min. The second-round PCR was performed using 2 µl of the first round reaction product in the same mixture described above but involving a monoplex primer for each G genotype. The PCR protocol was as follows: denaturation at 95°C for 9 min, 35 PCR cycles each consisting of 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min followed by an extension at 72°C for 10 min. 10 µl of PCR products were analyzed by 3% agarose gels (Panreac-Spain).

Monoplex Semi-nested RT-PCR for Human Rotavirus Group A P Genotyping

Human rotavirus group A P genotyping was performed using semi-nested RT-PCR according to Gentsch et al. with some modifications in the second semi-nested RT-PCR using monoplex primers instead of multiplex primers.[32] The first round RT-PCR amplified an 876 bp of the gene segment 4 (coding for VP4) using primers Con2 5′- ATTTCGGACCATTTATAACC-3′ and Con3 5′-TGGCTTCGCCATTTTATAGACA-3′. The second-round PCR was a monoplex semi-nested PCR and included the primer pair Con3 and a single P genotype-specific primer as follows: 1T-1(P[8] specific) 5′-TCTACTTGGATAACGTGC-3′, or 2T-1(P[4] specific) 5′-CTATTGTTAGAGGTTAGAGTC-3′, or 3T-1(P[6] specific) 5′-TGTTGATTAGTTGGATTCAA-3′ with a predicted product size of 346 bp, 483 bp, and 267 bp respectively.

The RT reaction with primers Con2 and Con3 was performed in a similar way that in G genotyping. The PCR mix for the first and second rounds of amplification was the same as that for the G genotyping except for annealing temperature of the second round PCR that was 44°C. 10 µl of PCR products were analyzed by 3% agarose gels (Panreac-Spain).

Statistical Analysis

McNemar's test was used to compare distributions of common G and P genotypes in the present study in both clinical specimens and raw sewage samples. Also, it was used to compare distributions of common G genotypes in the present study using monoplex nested RT-PCR with those of the multiplex nested RT-PCR in our previous study in both clinical specimens and raw sewage samples.[31]The McNemar's test was also used to compare distributions of common P genotypes in the present study using monoplex semi-nested RT-PCR with those of the multiplex semi-nested RT-PCR in our previous study in both clinical specimens and raw sewage samples.[31] P-value <0.05 was considered statistically significant.

RESULTS

Rotavirus Common G and P Genotypes among Rotavirus Positive VP6 Clinical Specimens

Using monoplex nested RT-PCR, rotavirus common G genotypes were detected in 57.60% (144/250) of the rotavirus positive VP6 clinical specimens. Thus 42.40% (106/250) of the rotavirus positive VP6 clinical specimens were untypeable for the common G genotypes. Furthermore, using monoplex semi-nested RT-PCR, rotavirus common P genotypes were detected in 92.80% (232/250) of the rotavirus positive VP6 clinical specimens. Thus, 7.20% (18/250) of the rotavirus positive VP6 clinical specimens were untypeable for the examined common P genotypes.

Notably, the most frequent G genotype was G1 (in 74 clinical specimens, 29.60%) followed by G3 (in 58 clinical specimens, 23.20%), G2 (in 7 specimens, 2.80%), and finally G4 (in 5 specimens, 2.00%). There were not any stool specimens, contained more than one G genotype (Figure 1). The most frequent P genotype was P[4] (in 104 clinical specimens, 41.60%) followed by P[8] (in 60 clinical specimens, 24.00%) and finally P[6] (in 51 clinical specimens, 20.40%). Mixed P genotypes were detected in 17 clinical specimens (6.80%), which 8 clinical specimens (3.20%) contained P[8] genotype mixed with P[6] genotype, 6 clinical specimens (2.40%) contained P[8] genotype mixed with P[4] genotype, and 3 clinical specimens (1.20%) contained P[8] genotype mixed with both P[4] and P[6] genotypes (Figure 2).

Rotavirus Common G and P Genotypes among Rotavirus Positive VP6 Raw Sewage Samples

Using monoplex nested RT-PCR, common G genotypes were detected in 70.83% (17/24) of the rotavirus positive VP6 raw sewage samples. Thus, 29.17% (7/24) of the rotavirus positive VP6 raw sewage samples were untypeable for the common G genotypes. Furthermore, using monoplex semi-nested RT-PCR, rotavirus common P genotypes were detected in 91.67% (22/24) of the rotavirus positive VP6 raw sewage samples. Thus, 8.33% (2/24) of the rotavirus positive VP6 raw sewage samples were untypeable for the common P genotypes.

The most frequent G genotype was G1 (in 9 raw sewage samples, 37.50%) followed by G3 (in 4 raw sewage samples, 16.67%), G2 and G4 (in 1 raw sewage sample for each of them, 4.17%). Mixed G genotypes (G1+G3) were detected in 2 raw sewage samples (8.33%) (Figure 3). The most frequent P genotype was P[4] (in 10 raw sewage samples, 41.67%) followed by P[8] (in 7 raw sewage samples, 29.17%), and finally P[6] (in 4 raw sewage samples, 16.67%). Mixed P genotypes (P[4]+P[8]) were detected in 1 raw sewage sample (4.17%) (Figure 4).

Comparative Distributions of Rotavirus Common G or Common P Genotypes in Both Clinical Specimens and Raw Sewage Samples in the Present Study Using Monoplex RT-PCR with those Using Multiplex RT-PCR in Our Previous Study

In clinical specimens, statistical analysis using McNemar's test indicated that there was extremely significant difference (the two-tailed P value was <0.0001) between the distribution of rotavirus common G genotypes in our present study using monoplex nested RT-PCR and the previous study using multiplex nested RT-PCR. Likewise, there was extremely significant difference (the two-tailed P value was 0.0077) between the distribution of rotavirus common P genotypes in our present study using monoplex semi-nested RT-PCR

and the previous study using multiplex semi-nested RT-PCR. Also, there was an extremely significant difference (the two-tailed P value was <0.0001) between common P and common G genotypes in our present study.

In raw sewage samples, there was the non-significant difference (the two-tailed P value was 0.1336) between the distribution of rotavirus common G genotypes in our present study using monoplex nested RT-PCR and the previous study using multiplex nested RT-PCR. Likewise, there was the non-significant difference (the two-tailed P value was 1) between the distribution of rotavirus common P genotypes in our present study using monoplex semi-nested RT-PCR and the previous study using multiplex semi-nested RT-PCR. Also, there was a non-significant difference (the two-tailed P value was 0.1306) between common P and common G genotypes in our present study.

DISCUSSION

Using monoplex nested RT-PCR of the present study, rotavirus common G genotypes were detected in clinical specimens with significantly higher frequency [57.60% (144/250)] than those detected by multiplex nested RT-PCR of our previous study [46.40% (116/250) which was reported by El-Senousy and co-workers.[31] All clinical specimens that were previously typeable for common G genotypes by multiplex nested RT-PCR (116 clinical specimens) were also typeable by monoplex nested RT-PCR assays of the present study. Additionally, the previously 134 (53.60%) clinical specimens that were negative for common G genotypes by multiplex nested RT-PCR when tested by monoplex nested RT-PCR of the present study, 28 (11.20%) clinical specimens could be genotyped for common G genotypes (9 clinical specimens with G1 genotype, 7 clinical specimens with G3 genotype, 7 clinical specimens with G2 genotype, and 5 clinical specimens with G4 genotype). The remaining 106 (42.40%) clinical specimens were untypeable for common G genotypes. Thus, G1 and G3 were detected with significantly higher frequency by the monoplex nested RT-PCR in the present study than those detected by multiplex nested RT-PCR in our previous study.[33] Although genotypes G2 and G4 were absent in the previous study, both of them could be detected in the present study with low percentages (2.80% and 2.00% respectively). This may return to the higher sensitivity of the monoplex nested RT-PCR which was used in this study about multiplex nested RT-PCR which was used in the previous study. On the other hand, in the study of Villena and co-workers when monoplex nested reaction was used, several additional raw sewage samples were G2 positive about the positive G2 samples detected using multiplex nested RT-PCR.[8] Also, using monoplex semi-nested RT-PCR of the present study, rotavirus common P genotypes (P[8], P[4], and P[6]) were detected in clinical specimens with significantly higher frequency [92.80% (232/250)] than those detected by multiplex semi-nested RT-PCR of our previous study [89.20% (223/250). All clinical specimens that were previously typeable for common P genotypes by multiplex semi-nested RT-PCR (223 clinical specimens) were also typeable by monoplex semi-nested RT-PCR assays of the present study. Additionally, the previously 27 (10.80%) clinical specimens that were negative for common P genotypes by multiplex semi-nested RT-PCR when tested by monoplex semi-nested RT-PCR, 9 (3.60%) clinical specimens could be genotyped for common P genotypes (4 clinical specimens with P[4] genotype, 3 clinical specimens with P[8] genotype, and 2 clinical specimens with P[6] genotype) while the remaining 18 (7.20%) clinical specimens were untypeable for common P genotypes. Thus, P[4], P[8], and P[6] were detected with significantly higher frequency by the monoplex semi-nested RT-PCR in the present study than those detected by multiplex semi-nested RT-PCR in our previous study.[33]

In raw sewage samples and using monoplex nested and semi-nested RT-PCR to detect G and P genotypes respectively, G1 genotype was detected in higher percentage (37.50%) than its percentage in the previous study which was published by El-Senousy and co-workers.[31] Genotypes G2 and G4 were detected with the same percentage for each of them (4.17%), while they were absent in the previous study which multiplex nested RT-PCR was used for detection. In the same time, P[4] genotype was detected in higher percentages (41.67%) than its percentage in the previous study which multiplex semi-nested RT-PCR was used for detection. The non-significant higher percentage of prevalence of G and P genotypes using monoplex nested and semi-nested RT-PCR may return to the high percentage of prevalence of G and P genotypes using multiplex nested and semi-nested RT-PCR about the total number of the studied samples. The nature of the samples which were raw sewage samples and may contain the high number of rotaviruses could be an important reason. Other types of samples especially treated effluents and drinking water samples may represent good candidates to study the difference in the efficiency of both monoplex and multiplex nested and semi-nested RT-PCR for investigation of rotavirus G and P genotypes as future research. The difference in the efficiency between the two methods may be very important when there will be a great need to chose a higher efficiency method to investigate human rotavirus G and P genotypes as the cause of infection in the gastrointestinal outbreaks.

The higher number of common typeable G and P clinical specimens and raw sewage samples in this study using monoplex nested and semi-nested RT-PCR in comparison to our previous study which multiplex nested and semi-nested RT-PCR assays were used for rotavirus genotyping may be due to the higher sensitivity of the monoplex nested RT-PCR than the multiplex nested RT-PCR in case of human rotavirus group

A G genotyping and also the higher sensitivity of the monoplex semi-nested RT-PCR than the multiplex semi-nested RT-PCR in case of human rotavirus group A P genotyping. It may return to the competition between the different primers in the construction of the specific strands using the dNTP's and the enzymes in case of both multiplexes nested RT-PCR and multiplex semi-nested RT-PCR. In the same time, there is no competition in case of both monoplex nested RT-PCR and monoplex semi-nested RT-PCR. The higher sensitivity of the monoplex RT-PCR than the multiplex RT-PCR was reported by a lot of authors.[33-37] Although multiplex RT-PCR has several advantages, including reduced labor and reagent costs and potentially faster detection, our results indicated its lower efficiency in comparison to the monoplex RT-PCR in case of human rotavirus G and P genotypes. These results may suggest the use of multiplex RT-PCR in case of screening of stool samples, however, monoplex RT-PCR could be used when investigation of some samples requires a higher sensitive test method.

Although the percentage of common G genotypes, when detected using monoplex nested RT-PCR, in the positive rotavirus VP6 diarrheal specimens (57.60%), was higher than their percentage when detected using multiplex nested RT-PCR in the same specimens (46.40%) which was previously reported, it is still an extremely significant difference between their frequency and the frequency of the common P genotypes in the same specimens when detected using monoplex semi-nested RT-PCR (92.80%).[31] This may confirm the significantly higher prevalence of common P genotypes than common G genotypes in Egyptian positive rotavirus VP6 diarrheal specimens collected from Abo El-Reech hospital in Greater Cairo which was previously reported by El-Senousy and co-workers who used multiplex semi-nested and nested RT-PCR for detection of common P and common G genotypes in the same positive rotavirus VP6 diarrheal specimens.[31] Higher prevalence of common P genotypes than common G genotypes was also observed in raw sewage samples collected from Greater Cairo using monoplex semi-nested and nested RT-PCR for detection of P and G genotypes respectively. This may confirm the higher prevalence of common P genotypes than common G genotypes which was observed in the same samples and was reported by El-Senousy and co-workers who used multiplex semi-nested and nested RT-PCR for detection of common P and common G genotypes respectively.[31] This higher prevalence of common P genotypes than common G genotypes in both clinical specimens and raw sewage samples in this study may confirm the suggestion of using recombinant subunit vaccine for human rotavirus group A using specific proteins of common P genotypes (P[4], P[6], and P[8]) in Egyptian community which was suggested by El-Senousy and co-workers.[31]

CONCLUSION

As general conclusions, the re-examination of the positive rotavirus VP6 stool specimens and positive raw sewage samples for common G and P genotypes using monoplex nested and semi-nested RT-PCR respectively was useful for increasing the number of typeable G and P specimens in this study in comparison to our previous study which multiplex nested and semi-nested RT-PCR respectively for common genotypes were used for genotyping.

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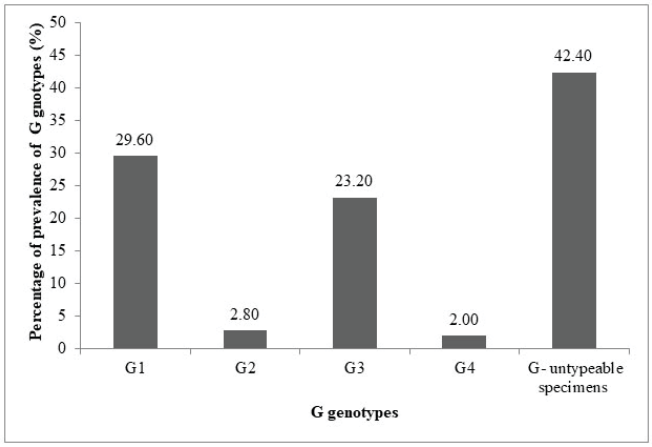

Figure 1: Distribution of rotavirus G genotypes among positive rotavirus VP6 clinical specimens using monoplex nested RT-PCR.

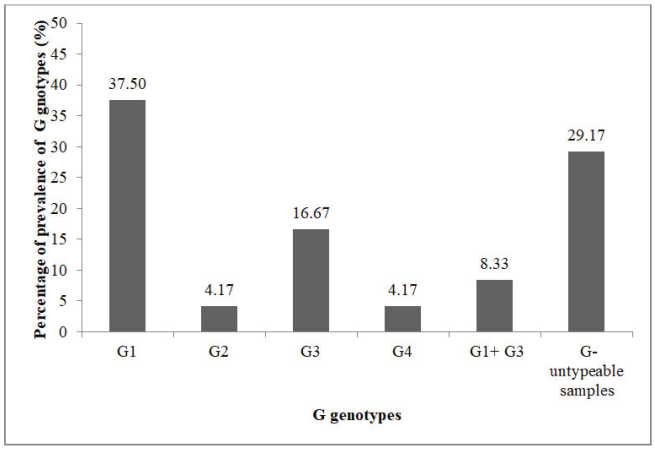

Figure 3: Distribution of rotavirus G genotypes among positive rotavirus VP6 raw sewage samples using monoplex nested RT-PCR.

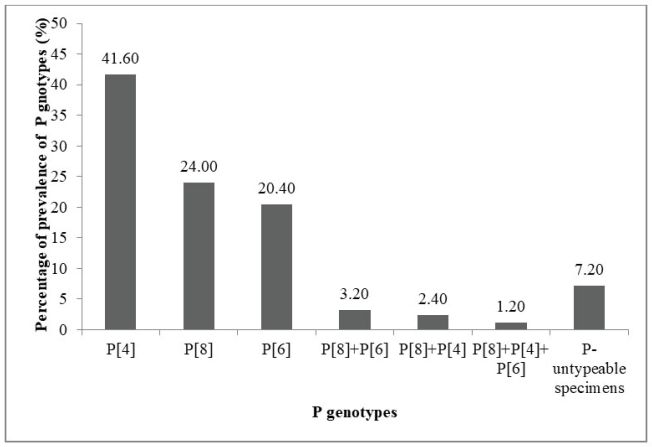

Figure 2: Distribution of rotavirus P genotypes among positive rotavirus VP6 clinical specimens using monoplex semi-nested RT-PCR.

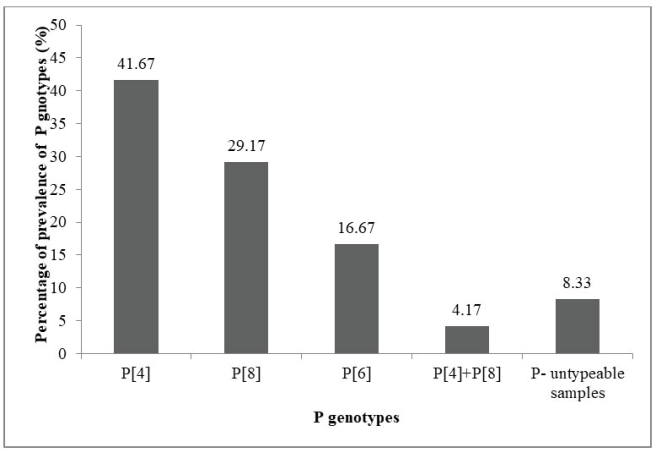

Figure 4: Distribution of rotavirus P genotypes among positive rotavirus VP6 raw sewage samples using monoplex semi-nested RT-PCR.