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Molecular Typing of *Rotaviruses* in Diarrheic Neonatal Calves

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Abstract

Rotavirus ribonucleic acid was extracted from 16 fecal samples of the serologically positive diarrheic calves using Latex agglutination test (LAT) and Immunochrmatographic assay (ICA). The extracted RNA was submitted to Reverse transcriptase polymerase chain reaction (RT-PCR) to detect VP7 and VP4 genes and the positive samples were 100% (16/16) and 81.25% (13/16), respectively. The amplified products were subjected to G and P-genotyping by semi-nested multiplex PCR using of G6, G8 and G10 genotyping and P1, P5 and P11 genotyping primers, respectively. G6 was detected in 10 (62.50%) of 16 samples and G10 was diagnosed in 5 (31.25%) of 16 samples and one (6.25%) sample did not react with any G primer used. P5 was detected in 9 (56.25%) of 16 samples, P11 was diagnosed in 3 (18.75%) of 16 samples, mixed infection with P5+P11 was observed in 1 (6.25%) of 16 samples and 3 (18.75%) samples did not react with any P primer used. G and P genotypes combination revealed that G6P5 was in 50% (8/16), G10P11 in 12.50% (2/16), G10P5 in 6.25% (1/16), G6P11 in 6.25% (1/16), G10 (P5+P11) in 6.25% (1/16), G6P? in 6.25% (1/16), G10P? in 6.25% (1/16), and G?P? in 6.25% (1/16). These results suggest that the detected genotypes can used as dominant strains for the formulation of an appropriate vaccine against BRV in Assiut Governorate. In conclusion, RT-PCR and Semi-nested multiplex PCR can used as rapid and confirmatory test for detection of nucleic acid and genotypes of *Rotavirus*, G and P genotypes combination in the present study revealed that G6P5, G6P11, G10P5 and G10P11 were circulating genotypes in bovine population in Assiut governorate. G6P5 strain was the most common of all strain diagnosed in other fecal samples. The presence of various combinations of G and P genotypes among field isolates of BRV suggests that genetic reassortment frequently occurred between viral strains with genes encoding different G and P genotypes. Finally, presence of different genotypes of *Rotaviruses* emphasizes their simultaneous monitoring in animals for the development and optimization of *Rotavirus* vaccines.

KEYWORDS
Bovine *Rotavirus*, RNA, RT-PCR, G and P Genotyping.

INTRODUCTION

Rotaviruses are important etiological agents of acute viral enteritis in neonatal calves (Zaitoun *et al*., 2018). *Rotavirus* is a non-enveloped virus of genus *Rotavirus*, belongs to family Reoviridae and has a genome of 11 segments of double stranded ribonucleic acid (dsRNA) that is enclosed within a triple layered capsid protein. *Rotavirus* encodes six viral structural proteins (VP) (VP1–VP4, VP6 and VP7) and six non-structural proteins (NSP) (NSP1–NSP6) (Murphy *et al*., 1999). Based on VP6, *Rotaviruses* are classified into ten serogroups (A-J) (Babalola, 2020; Hossain *et al*., 2020). *Rotavirus* has two neutralization proteins, VP4 (protease-sensitive protein) and VP7 (glycoprotein) on its outer capsid, these two proteins define the P and G genotypes, respectively. *Rotaviruses* are classified into 36 G genotypes and 51 P genotypes based on nucleotide sequences of VP7 and VP4 genes, respectively by *Rotavirus* Classification Working Group (Sawant *et al*., 2020; Elkady *et al*., 2021). *Rotavirus* genotypes G6, G8 and G10 (associated with P1, P5 and P11) are the most epidemiologically important genotypes around the world (Fritzen *et al*., 2019; Bertoni *et al*., 2020; Elkady *et al*., 2021). *Rotaviruses* are active everywhere, and hence more data on G and P types circulating parts of the world will be valuable for those searching for formulation of an adequate vaccine (Anamul *et al*., 2015). Genotyping used to identify genotypes of *Rotavirus* isolates of diarrheic calves. There is no information on G and P diversity of BRV circulating locally in Assiut governorate, therefore the current work aimed to detecting and typing of *Rotaviruses*' genomes in fecal samples of enteric serologically positive calves by using polymerase chain reaction (PCR) based typing assays.

MATERIALS AND METHODS

Sampling

During the period of investigation, from January 2018 to November 2019, a total of 16 fecal samples

Were collected from 15 cattle and 1 buffalo enteric neonatal

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calves (aged from 3 - 60 days) that admitted to the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Assiut University. Fecal samples were serologically positive for Rotavirus infection by Latex agglutination test (LAT) and Immunochrmatographic assay (ICA). The study was conducted according to the ethical guidelines for Animal Welfare Act at the Faculty of Veterinary Medicine, Assiut University, Egypt.

Molecular diagnosis

Viral RNA extraction

For VP4 and VP7 genes of BRV amplification, the viral RNA was extracted from 16 serologically positive fecal samples by using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted RNA was stored at -80ºC until further use.

Primers

The specificities of the selected primers (Invitrogen, Thermo Fisher Scientific, Germany) used in the present study for VP7 and VP4 genes, and G and P genotyping assays have been evaluated previously (Isegawa *et al*., 1993; Falcone *et al*., 1999). Sequences of primers and their positions in genomic segment are presented in (Tables 1 and 2).

Rotavirus detection by RT-PCR of VP7 and VP4 genes

RT-PCR was performed to detect *Rotavirus* gene in the fecal samples. Reaction was carried out by conventional method using One-Step RT-PCR Kit (Qiagen, Hilden, Germany with pairs of forward and reverse amplification primers (VP7-F/ VP7-R and VP4-F/ VP4-R) to amplify full-length of VP7 and VP4 gene, respectively. All extracted RNAs were denatured at 97°C for 3 min and immediately placed on ice (Youssef, 2017). The denatured dsRNA (10 µl) was then added to the reaction mixture, consisting of 5 µl Qiagen One-Step RT-PCR buffer, 5x, 1µl of a deoxynucleoside triphosphate (dNTP) mixture (each dNTP at a concentration of 400 µM), 2 µl of each primer (20 pmol), 1µl Qiagen One-Step RT-PCR Enzyme mix and 4 µl RNase free water in a final volume of 25 µl, Then, One-Step RT-PCR was performed by using Qiagen OneStep RT-PCR Kit (Qiagen, Hilden, Germany) according the manufacture's, the annealing temperature of VP7 gene was set at 52°C and the annealing temperature of VP4 gene was set at 48°C.

Determination of G genotypes by semi-nested multiplex PCR

RT-PCR products of VP7 gene were submitted to a second round of amplification for G genotyping, respectively by the following. An aliquot of 4 µl of diluted (1:30) DNA products was added to a reaction mixture consisting of 12.5 µl COSMO PCR RED Master Mix (Willowfort, Biocity Pennyfoot, United Kingdom), 2 µl (20 pmol) of VP7 upstream primer, 2 µl (20 pmol) of each primer specific for (G6, G8 and G10) and RNase-free water to a final volume of 25 µl. The second round of amplification consisted of 35 cycles of 5 min at 95°C, 45 sec at 95°C, 45 sec at 54°C, and 1 min at 72°C, followed by a final extension at 72°C for 10 min.

Determination of p genotypes by semi-nested multiplex PCR

RT-PCR products of VP4 gene submitted to a second round

Table 1. Primers sequences for amplification of VP7 and VP4 genes of BRV by RT-PCR

Gene	Primer	Sequence (5' to 3')	Position	Product size (bp)	References
VP7	Bov9Com5 (Forward)	GGCTTTAAAAGAGAGAATTTCCGTTTGG	1-28	1062	Isegawa *et al.* (1993) and Falcone *et al.* (1999)
	Bov9Com3 (Reverse)	GGTCACATCATACAACTCTAATCT	1039 - 1062		
VP4	Bov4Com5 (Forward)	TTCATTATTGGGACGATTCACA	1064 - 1085	856	
	Bov4Com3 (Reverse)	CAACCGCAGCGGATATATCATC	1897 – 1918		

Table 2. Primers sequences for amplification of G and P genotyping of BRV by semi-nested multiplex PCR

Gene	Genotype	Primer	Sequence (5' to 3')	Position	Product size (bp)	References
VP7		Bov9Com5 (Forward)	GGCTTTAAAAGAGAGAATTTCCGTTTGG	1-28		Isegawa *et al.*, 1993 and Falcone *et al.*, 1999
	G6	G6 (Reverse)	CTAGTTCCTGTGTAGAATC	499 - 481	500	
	G8	G8 (Reverse)	CGGTTCCGGATTAGACAC	273 - 256	274	
	G10	G10 (Reverse)	TTCAGCCGTTGCGACTTC	714 – 697	715	
VP4		Bov4Com5 (Forward)	TTCATTATTGGGACGATTCACA	1064 - 1085		
	P1	P1 (Reverse)	TTAAATTCATCTCTTAGTTCTC	1505 - 1526	463	
	P5	P5 (Reverse)	GGCCGCATCGGATAAAGAGTCC	1704 - 1725	662	
	P11	P11 (Reverse)	TGCCTCATAATATTGTTGGTCT	1377 – 1398	335	

of amplification for p genotyping. The P genotyping assay was performed by the same protocol followed for the characterization of G genotypes but with 2 µl (20 pmol) of VP4 upstream primer and 2 µl (20 pmol) of each primer specific for (P1, P5 and P11).

Analysis and detection of PCR products

PCR products were analyzed gel electrophoresis at 110 V for 70 mins in 1.5% agarose gel containing ethidium bromide (10 mg/ml) in 1x Tris-Acetate- EDTA buffer and photographed by a gel documentation system (BDA digital Biometra, Germany)

RESULTS

RT-PCR analysis

In the present study, 16 serologically positive fecal samples of enteric calves were examined by using RT-PCR for detection of *Rotavirus* nucleic acid. 16 (100%) and 13 (81.25%) of 16 samples were positive by VP7 and VP4, respectively. The specific band showed at 1062 bp after amplification of VP7 gene (Fig. 1) and another specific band showed at 856 bp after amplification of VP4 gene of BRV (Fig. 2).

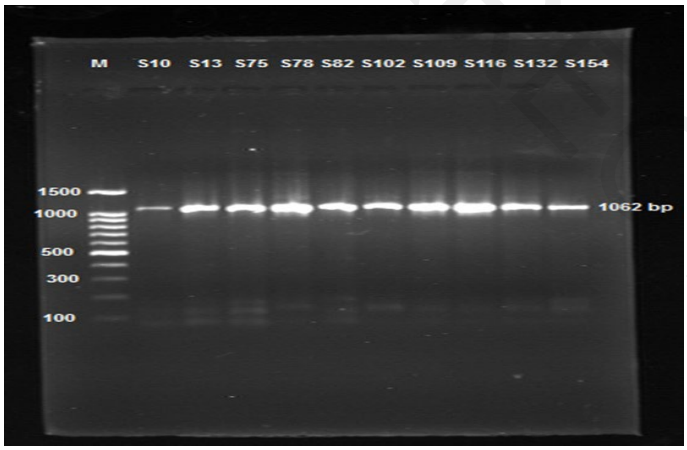

Figure 1. Agarose gel electrophoresis of RT-PCR after amplification of VP7 gene of BRV. Lane M: DNA Marker of 100 bp. Lanes S10, S13, S75, S78, S82, S102, S109, S116, S132 & S154: Positive samples with amplified product at 1062 bp.

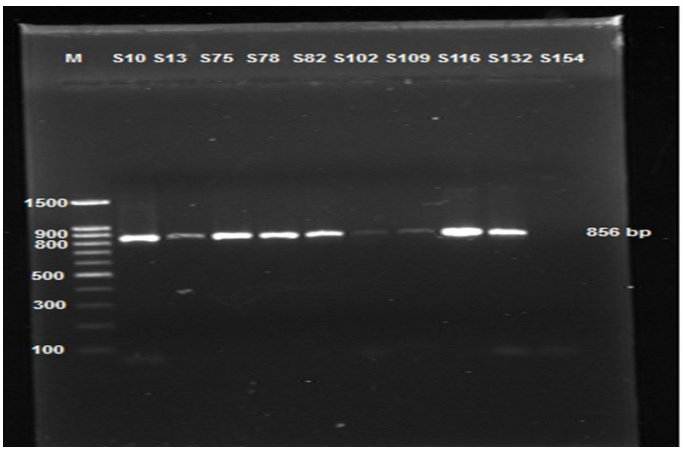

Figure 2. Agarose gel electrophoresis of RT-PCR after amplification of VP4 gene of BRV. Lane M: DNA Marker of 100bp. Lanes S10, S13, S75, S78, S82, S102, S109, S116& S132: Positive samples with amplified product at 856bp. 7 gene and didn't react with VP4 gene. Lane S154: Positive fecal sample by VP.

G genotyping

In this this study, 16 molecularly positive fecal samples of enteric calves by VP7 gene were examined by using semi-nested multiplex PCR for detection of G6, G8 and G10 genotypes. 15 (93.75%) out of 16 samples were positive for G-Genotyping. G6 and G10 were detected but G8 didn't be diagnosed in our result. The specific band of G6 showed at 500 bp and specific band of G10 showed at 715 bp after amplification of DNA product of VP7 gene (Fig. 3). G6 was detected in 10 (62.50%) out of 16 samples and G10 was diagnosed in 5 (31.25%) out of 16 samples and one (6.25%) sample did not react with any G primer used. G6 was the most common genotype of BRV in diagnosed fecal samples of enteric calves.

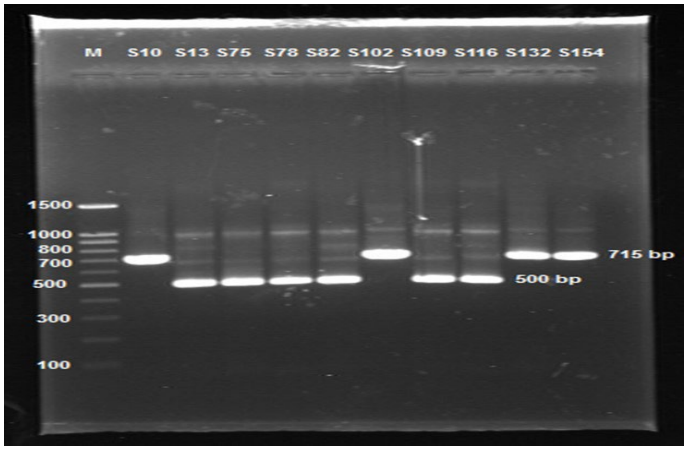

Figure 3. Agarose gel electrophoresis of G-Genotyping of BRV after amplification of DNA product of VP7 gene. Lane M: DNA Marker of 100bp. Lanes S10, S102, S132 & S154: Positive samples with amplified product of G10 at 715 bp.

P genotyping

In the current study, 16 molecularly positive fecal samples of enteric calves were diagnosed by using semi-nested multiplex PCR for detection of P1, P5 and P11 genotypes. 13 (81.25%) out of 16 samples were positive for P-Genotyping. P5 and P11 were diagnosed but P1 didn't be detected in our result. The specific band of P5 showed at 662 bp while specific band of P11 showed at 335 bp after amplification of DNA product of VP4 gene (Fig. 4). P5 was detected in 9 (56.25%) out of 16 samples, P11 was diagnosed in 3 (18.75%) out of 16 samples, mixed infection with P5+P11 was observed in 1 (6.25%) out of 16 samples and 3 (18.75%) samples did not react with any P primer used. P5 was the most common genotype of BRV in diagnosed fecal samples of enteric calves.

G and P genotypes combination

In the present study, 16 RT-PCR positive fecal specimens of enteric calves were examined by semi-nested multiplex PCR for G and P genotyping. G and P genotypes combination revealed that G6P5 was in 50% (8/16), G10P11 in 12.50% (2/16), G10P5 in 6.25% (1/16), G6P11 in 6.25% (1/16), G10(P5+P11) in 6.25% (1/16), G6P? in 6.25% (1/16), G10P? in 6.25% (1/16), and G?P? in 6.25% (1/16). G6P5 strain was the most common of all BRV strains diagnosed in other fecal samples (Table 3).

DISCUSSION

Currently, 16 fecal samples of investigated enteric calves were molecularly diagnosed by RT-PCR for detection of *Rotavirus* infection and revealed 16 (100%) and 13 (81.25%) of 16 samples were positive by VP7 and VP4 genes, respectively. The obtained specific viral amplicons with sizes of 1062 bp of amplified VP7 gene and 856 bp of amplified VP4 gene were estimated con-

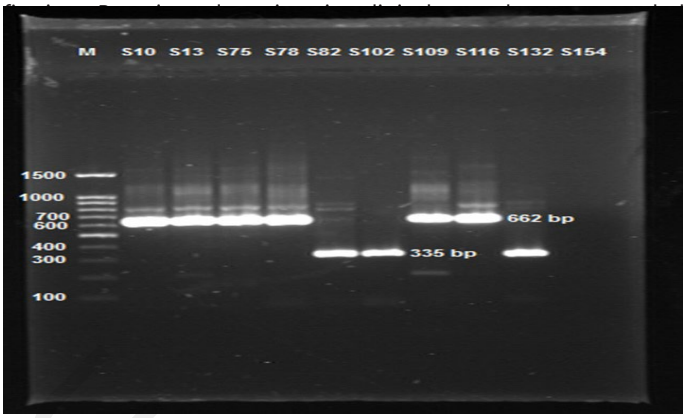

Figure 4. Agarose gel electrophoresis of P-Genotyping of BRV after amplification of DNA product of VP4 gene. Lane M: DNA Marker of 100bp. Lanes S10, S13, S75, S78, S109 & S116: Positive samples with amplified product of P5 at662 bp. Lanes S82, S102 & S132: Positive samples with amplified product of P11 at 335 bp. Lane S154: P? (Not determined).

Table 3. Distribution of various G and P genotypes combination of BRV in 16 molecularly positive enteric calves.

G and P Genotypes	No.	%
G6P5	8	50
G10P11	2	12.5
G10P5	1	6.25
G6P11	1	6.25
G10(P5+P11)	1	6.25
G6P?	1	6.25
G10P?	1	6.25
G?P?	1	6.25

? = Untyped

previously by Falcone *et al*. (1999); De Martino *et al*. (2011); Hassan (2014); Deswal *et al*. (2015) and Fritzen *et al*. (2019). In the obtained result, lower number of positive fecal samples by VP4 gene than VP7 gene. This may be attributed to VP4 RT-PCR was less sensitive than VP7 RT-PCR (Doorn *et al*., 2009). Regarding to G-genotyping of BRV in present study, 15 (93.75%) out of 16 fecal samples were positive. G6 and G10 were detected but G8 didn't be diagnosed. The obtained specific band of G6 showed at 500 bp and specific band of G10 showed at 715 bp after amplification of DNA product of VP7 gene as reported previously by Falcone *et al*. (1999); Tarunkumar (2009) and Gill *et al*. (2017). In the current study, G6 was diagnosed in 10 (62.50%) out of 16 samples, G10 was detected in 5 (31.25%) out of 16 samples and one (6.25%) sample was untyped. G6 was the most common genotype of BRV in diagnosed fecal samples of enteric calves that similar with the distribution of BRV genotypes elsewhere in the world, where low prevalence rate of the G10 genotype and higher prevalence of the G6 genotype have been reported by Falcone *et al*. (1999(; Alfieri *et al*. (2004); Pisanelli *et al*. (2005); Reidy *et al*. (2006); Mayameii *et al*. (2007); Howe *et al*. (2008); Monini *et al*. (2008) and Mohamed *et al*. (2017) who mentioned that G6 genotype was predominant than G10 genotype. Contrariwise, G10 was higher than G6 in diagnosed fecal samples of enteric calves that obtained by Tarunkumar (2009) and Sawant *et al*. (2020) who found that G10 was higher than G6 genotype. G8 genotype was not detected in any of fecal samples; this finding was reported by Tarunkumar (2009). This may be attributed to BRV G8 genotype had been reported to be rare in some area and G8 is one of the less common genotypes among bovine *Rotaviruses*. However, it could be considered a diarrheal pathogen diagnosed with lower frequency in calves (Tarunkumar, 2009). According to P-genotyping, 13 (81.25%) out of 16 fecal samples were positive. P5 and P11 were diagnosed but P1 didn't be detected in our study. The obtained specific band of P5 showed at 662 bp while specific band of P11 showed at 335 bp after amplification of DNA product of VP4 gene as recorded previously by Falcone *et al*. (1999) and Hassan (2014). In the current study, P5 was diagnosed in 9 (56.25%) out of 16 samples, P11 was diagnosed in 3 (18.75%) out of 16 samples, mixed infection with P5+P11 was noted in 1 (6.25%) out of 16 samples and 3 (18.75%) samples were untyped. P5 was the most common genotype of BRV in diagnosed fecal samples of enteric calves which similar with the distribution of BRV genotypes elsewhere in the world, where low prevalence rate of P11 genotype and higher prevalence of P5 genotype have been reported by Reidy *et al*. (2006Contrariwise, P11 was higher than P5 in diagnosed fecal samples of enteric calves that obtained by Falcone *et al*. (1999); Hassan (2014) and Hassine-Zaafrane *et al*. (2014). P1 genotype was not detected in any of fecal samples; similar finding was reported by Falcone *et al*. (1999). This may be attributed to that BRV P1 strain is one of the less common genotype s among bovine *Rotaviruses*. Mixed *Rotavirus* strains infections detected in this study P5+P11 were among those P genotypes frequently observed in BRVA strains; similar result was recorded by Barreiros *et al*. (2004) and Caruzo *et al*. (2010). This may be due to increase in immunity to variant *Rotavirus* genotypes may lead to formation of reassortants (Barreiros *et al*., 2004). Thus, similarity and contradiction of this finding suggests that G6, G10, P5 and P11 genotypes predominance may be restricted as per geographical location The present study suggests that the vaccine strains in order to prevent *Rotavirus* infection in enteric calves it should include G6, G10, P5 and P11 genotypes. Our results might help in future studies on *Rotaviruses* in Egypt especially in Assiut.

Regarding to genetic combination of G and P types in the current study, out of 16 RT-PCR positive fecal specimens of enteric calves, G6P5 combination was most detected in 8 (50%) samples. G10P11 was diagnosed in 2 samples (12.50%) and G10P5 was also detected in 1 sample (6.25%) followed by one sample (6.25%) was G6P11. This similar finding was recorded in other studies elucidated by De Brito *et al*. (2000); Rodríguez-Limas *et al*. (2009); De Martino *et al*. (2011); Pourasgari *et al*. (2016); Gill *et al*. (2017); Mohamed *et al*. (2017); Rondelli *et al*. (2018) and Fritzen *et al*. (2019) who found that G6P5, G6P11, G10P5 and G10P11 strains of *Rotavirus* affect enteric calves. Our study indicated that G6P5 was the predominate strain of *Rotavirus* affect enteric calves. G6P5 strain was the highest of all strains detected in other fecal samples in this study. This finding was similar to studies of De Brito *et al*. (2000); Alfieri *et al*. (2004); Barreiros *et al*. (2004); Caruzo *et al*. (2010); De Martino *et al*. (2011); Collins *et al*. (2014); Pourasgari *et al*. (2016); Rondelli *et al*. (2018) and Fritzen *et al*. (2019) who reported G6P5 as the most dominant genotypes combination of *Rotavirus* strain in examined infected enteric calves. However, the obtained finding is in contrary to studies of Hassine-Zaafrane *et al*. (2014) and Gill *et al*. (2017) who found that G6P11 was the most predominant strain, and to Rodríguez-Limas *et al*. (2009); Beg *et al*. (2010); Hassan (2014who concluded that G10P11 was the most common *Rotavirus* strain in investigated enteric calves. In the current study, one sample (6.25%) showed evidence of mixture of two strains with G10 (P5+P11) which indicates that a mixed infection with more than one *Rotavirus* can cause enteritis in calves (Howe *et al*., 2008; Caruzo *et al*., 2010; Pourasgar *et al*., 2016). In current result, some samples were partially and completely untyped such as G6P? in one sample (6.25%), G10P? in one sample (6.25%) and G?P? in one sample (6.25%). Such similar finding was observed by other studies mentioned by Falcone *et al*. (1999); Hassan (2014); Hassine-Zaafrane *et al*. (2014); Pourasgari *et al*. (2016); Rondelli *et al*. (2018) and Sawant *et al*. (2020). In the present study, the accuracies of G- and P-Genotyping analysis performed also resulted in a very limited number of untyped strains. This number was extremely low compared to the number obtained in other studies recorded by Hassine-Zaafrane *et al*. (2014); Pourasgari *et al*. (2016) and Sawant *et al*. (2020). The PCR-based genotyping used was further confirmed method to be a useful epidemiological tool for investigating strain diversity, although it has gaps in encompassing novel or uncommon *Rotavirus* genotypes (Falcone *et al*., 1999; Hassan, 2014; Hassine-Zaafrane *et al*., 2014; Pourasgari

et al., 2016). From the present study, it is very important to establish an epidemiologic surveillance and control of BRV infection, as it has been shown that protection between genotypes is limited and available vaccines sometimes do not provide cross-protection (Rodríguez-Limas *et al*., 2009; De Brito *et al*., 2000).

CONCLUSION

RT-PCR and Semi-nested multiplex PCR can be used as confirmatory test for detection of nucleic acid and genotypes of *Rotavirus*, respectively. G and P genotypes combination in the present study revealed that G6P5, G6P11, G10P5 and G10P11 were circulating genotypes in bovine population in Assiut governorate. G6P5 strain was the most common of all strain diagnosed in the fecal samples. The presence of various combinations of G and P genotypes among field isolates of BRV suggests that genetic reassortment frequently occurred between viral strains with genes encoding different G and P genotypes. Finally, presence of different genotypes of *Rotaviruses* emphasizes their simultaneous monitoring in animals for the development and optimization of *Rotavirus* vaccines.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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