

Original Research Article

<https://doi.org/10.20546/ijcmas.2019.809.296>

Molecular Detection of Group A Rotaviruses from the Bhandara and Chandrapur District of Maharashtra State, India

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ABSTRACT

Animal *Rotaviruses* (RVs) are considered as major pathogen threats to humans and livestock due to neonatal mortality and possibility of interspecies transmission and exchange of genomic materials from human to animal species and vice versa. There are eight groups of rotaviruses (A to H), among them mostly Group A mostly causes diarrhea in neonates and infants in humans and animals especially in cattle and buffalo worldwide. The present study was carried out to emphasize for molecular detection of circulating rotaviruses in Bhandara and Chandrapur districts of Maharashtra state in India. Ninety two (92) fecal samples collected from diarrheic and non diarrheic bovine calves (39 cow calves and 53 buffalo calves) were analyzed by conventional latex agglutination test (LAT) and amplification of VP6, VP7 and VP4 gene by RT-PCR and further analysis by G and P Typing PCR. From ninety two samples, ten samples were found positive by LAT, out of ten LAT positive samples, four samples showed expected 309 bp amplicon for group specific VP6 gene. The VP4 and VP7 RT-PCR showed 864 bp and 1062 bp amplicons specific for VP4 and VP7 respectively in same two fecal out of ten positive samples. The G-typing PCR showed the G10 type strain is circulating whereas P type was untypable. Hence the present study concludes, the presence of group A Rotaviruses of G10 Type in Bhandara district and untypable rotaviruses in Chandrapur district of Vidharbha region of Maharashtra state in India.

Keywords

Rotaviruses, Latex agglutination test, RT-PCR, VP6, VP4 and VP7

Article Info

Accepted:

24 August 2019

Available Online:

10 September 2019

Introduction

The *Rotavirus* is zoonotic important enteric viral pathogen suspected in wide host range in mammals and birds. It was reported to cross

species transmission. The rotavirus led to diarrhea, emaciation and death in infants and calves. Rotavirus infects villous structure of small intestines where it lodges and multiplication occurred. The rotaviruses

belongs to family *Reoviridae*, subfamily *Sedoreovirinae* of genus *Rotavirus*, which further composed of nine species namely *Rotavirus A*, *Rotavirus B*, *Rotavirus C*, *Rotavirus D*, *Rotavirus E*, *Rotavirus F*, *Rotavirus G*, *Rotavirus H*, *Rotavirus I* (ICTV taxonomy (2018b)). Rotaviruses are structurally icosahedral, triple layered and non-envelope viruses.

The genome is composed of 11 segments of double stranded RNA (ds RNA), which encode six structural (VP1, VP2, VP3, VP4, VP5, VP6 and VP7) and six non structural proteins (NSP1-NSP6). The VP6 protein is group specific inner capsid protein has high degree of nucleotide sequence conservation in different viral strains. The two outer layered proteins VP4 and VP7 are classified into P types and G types respectively. These two proteins elicit neutralizing antibody response. Due to segmented nature of virus the two outer proteins VP4 and VP7 can segregate independently during reassortment, owing to which different G types and P types combinations could be found in co-circulating rotaviruses.

Rotavirus infection could be mild, moderate or severe. It varies from no symptoms to severe diarrhea, emaciation, body weight loss, no body weight gain in animals. It also causes economical burden to family due to treatment cost on the rotavirus disease, there is scanty data available in case of bovine rotavirus in Vidharbha region of Maharashtra. Farmers of Vidharbha region of Maharashtra are not much aware of the rotavirus infection in animals and human and cross transmission of viral infection. Hence, the study was planned to characterize and to study prevalence of bovine rotavirus circulating in Bhandara and Chandrapur district of Vidharbha region in Maharashtra where animal husbandry is well adopted and calf diarrhea and neonatal mortality is reported.

Materials and Methods

Collection of samples

Samples were collected from goshala, commercial dairy farms and unorganized farms in the months of August to September 2018. The total, ninety two (92) samples from both districts, forty four (44) from Bhandara (MS) and forty eight (48) from Chandrapur (MS) from the calf age 3 days to 90 days from diarrheic and non diarrheic bovine calves (39 cow calves and 53 buffalo calves). The fecal samples were collected from calves with diarrhea having consistency like semisolid, liquid, watery, and color greenish, yellowish, whitish, bloody diarrhea and non diarrheic at the time of samples collection but calves with history of frequently diarrhea before. Fecal samples were collected in sterile cotton swab from rectum of calves and kept on ice packs in cold chain transport box brought to Department of Veterinary Microbiology in Nagpur Veterinary College, Nagpur and kept in -20°C for further investigation.

Faecal suspension preparation

A 10 % fecal suspension was prepared in 2 ml microcentrifuge tube in 1X phosphate buffered saline (PBS, pH 7.4) and clarified by centrifugation at 1000 g for 10 minutes in a refrigerated centrifuge (Biofuge primoR, Thermo Fischer Scientific, Germany) at 4°C . The supernatant was transferred to fresh tube as clarified fecal/viral suspension and used as basic material for extraction of rotavirus RNA. The supernatant was stored at -70°C till further use.

Screening of samples by conventional method: Latex agglutination test

Latex agglutination test was carried out using the commercially available HiRotavirus Latex Test Kit (Cat. No LK08-50NO Himedia, India)

as per manufacturer's instructions. Ten percent fecal suspension was prepared into in 1.0 millilitre extraction buffer and allowed to settle down for larger particle and centrifuge for 1000g for 10 mins. The 50µl inoculum was placed on LAT card wells and to this 20 µl mixed rotavirus latex reagent (LK08a) to one well and 20 µl of rotavirus control reagent (LK08b) to other well was added. The contents of each well were mixed by using a separate mixing stick for each well and spread thoroughly in well by gentle rocking the card and agglutination within two minutes was recorded.

Molecular detection of rotaviruses

RNA-Extraction and cDNA Synthesis

Total RNA was extracted from ten latex agglutination positive samples by Trizol Reagent LS (Cat.No.10296010, Invitrogen, USA) as per manufacturer's instructions with some modifications. All extracted RNA samples were quantified by Nanodrops machine (Thermo fisher Scientific, USA) and purity of RNA was checked by A260/280 and concentration checked in nanogram/microlitre(ng/µl).The cDNA Synthesis was performed using superscript III First-Strand synthesis system (18080-051) kit protocol with some modification. The reaction was performed in 0.2ml PCR tubes as followed 1µl of dNTPs (10Mm), 2µl of DMSO, 2 µl of Random hexamer (50ng/ µl) and 7µl RNA (~ 1-2 µg) was added and mixture was incubated at 95⁰c for 5 min and snap chilled in ice. To this mix, 2.5 µl of 10 X RT Buffer, 5 µL of MgCl₂ (25 mM), 2.5 µl of DTT (0.1M), 1 µl of DMSO and 1 µl of Superscript enzyme (200U/ µl) was added, mixed and briefly short spinned. The reaction was carried out as, 25⁰C for 10 min, 50⁰C for 50 min followed by 85⁰C for 5 min. After completion of this reaction 1 µl of RNase H (2U/ µl) was added and further incubated at

37⁰C for 20 min. The cDNA was stored at -20⁰C till further use.

Detection of Bovine Rotaviruses by Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

The amplification of VP6 for Rotaviruses

The published Primer forward - Rot3 F-5'AAA GAT GCT AGG GAC AAA ATT G3' and reverse- Rot5 R-5' TTC AGA TTG TGG AGC TAT TCC A3' was used in the study (Elschner *et al.*, 2002). The PCR was carried out in 0.2 ml PCR tubes in 25 µl total reactions volume containing 12.5 µl 2X PCR Master Mix(Cat.No.PGK017-A,Puregene Genetix,India),2µl of forward primer (10pmol/ µl) and 2µl of reverse primer(10pmol/ µl), 3µl of cDNA and 5.5µl of nuclease free water. The PCR was carried out in cyclic condition initial denaturation of 95⁰C for 5 minutes; followed by 40 cycles of denaturation at 95⁰C for1min, annealing for 52⁰C for 1min, extension for 72⁰ C for 1 min and final extension on 72⁰C for 10 min. The PCR products were resolved in 1.5% agarose gel electrophoresis at 60 V for 1 hour in 0.5X TBE buffer in Electrophoresis unit (Banglore Genei,India)and documented using Geldoc system (Gel.ProCCD116, BioZen Laboratories, India).

The amplification of VP7 gene and VP4 gene for Rotaviruses

The amplification of VP7 gene was carried out using the published primer (Taniguchi *et.al.*, 1992), targeting VP7 gene: forward -5'-GGC TTT AAA AGA GAG AAT TTC CGT CTG G-3' and reverse-5'-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3'. The PCR was carried out in 0.2 ml PCR tubes containing reaction mixture of 10µl PCR Master Mix (2X)(Puregene genetix Cat.No.PGK017-A,India),2µl of forward primer (10pmol/ µl)

and 2µl of reverse primer(10pmol/ µl), 1.5 µl of cDNA, 1µl of DTT (0.1M) and 3.5 µl of nuclease free water for the 20 µl total reactions. The PCR was carried out with cycling conditions as initial denaturation of 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 sec; annealing at 55 °C for 1min and extension at 72°C for 2 min; and final extension at 72°C for 10 min.The PCR products were resolved in 1% agarose gel electrophoresis at 60 V for 1 hour in 0.5 X TBE buffer in Electrophoresis unit (Banglore Genei, India) viewed and documented using Geldoc system (ProCCD116 BioZen Laboratories, India.).

The amplification of VP4 gene was carried out using the published primer sequence Isegawa *et. al.*,(1993), forward 5'-TTC ATT ATT GGG ACG ATT CAC A-3' and reverse primer 5'-CAA CCG CAG CTG ATA TAT CAT C-3'.The PCR was carried out in 0.2 ml PCR tubes containing reaction mixture as above mentioned reaction.The PCR was carried out with cyclic condition as initial denaturation of 95°C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 sec; annealing at 52 °C for 1min, extension at 72°C for 1 minute and the final extension was carried out at 72°C for 10 min. The PCR products were resolved in 1% agarose gel in 0.5 X TBE buffer in Gelectrophoresis unit (Banglore Genei,India) viewed and documented using Geldoc system (ProCCD116 BioZen Laboratories, India).

G and P genotyping

The amplified positive PCR products for VP7 gene were further processed for G-typing. The amplified products were diluted 1:50 in nuclease free water (NFW) and from diluted product 3 µl was used for typing reaction. The reaction was prepared in 0.2 ml PCR tube with 5µl of reverse primer(10pmol/µl) reverse-5'-GGT CAC ATC ATA CAA TTC TAA TCT

AAG-3' and G-typing cocktail each published primer 1 µl (G3,G5,G6,G8 and G10),the mixture of 25.0 µl PCR Master Mix(2X)(Puregene genetixCat.No.PGK017-A,India), 2µl of DTT(0.1M) ;10.0µl of nuclease free water adjust the 50 µl total reactions. All components were mixed properly by tapping and briefly spunned. The PCR tube kept in thermocycler and cycling condition was set same for VP7 amplification.

The amplified positive PCR products for VP4 gene were processed for P-typing. The positive amplified PCR products were diluted 1:50 and from diluted product 3µl was used for typing. The reaction was prepared in 0.2 ml PCR tube with 2µl of forward primer(10pmol/ µl Forward -5'-TTC ATT ATT GGG ACG ATT CAC A-3 and P-typing cocktail each published primer 1 µl of P[1], P[5] and P[11]the mixture of 10.0 µl PCR Master Mix(2X) (Puregene genetix Cat.No.PGK017-A,India),1 µl MgCl₂ (50mM), 1µl of DTT (0.1M) and 1.0 µl of nuclease free water in the 20 µl total reactions. All components were mixed properly by tapping with briefly spunned. The PCR tubes were kept in thermocycler and cycling condition was set same VP4 amplification–typing and P-typing amplified PCR products were resolved in a 1% agarose gel in 0.5 X TBE buffer in Gelectrophoresis unit (Banglore Genei,India) viewed and documented using Geldoc system (ProCCD116 BioZen Laboratories, India).

Results and Discussion

Bovine *Rotavirus* infection leads to morbidity and mortality in calves of age between first week to 24 weeks that result into substantial economic losses to dairy industry throughout the world and immensely in developing and undeveloped countries. In Maharashtra State, in Vidharbha region, dairy industry is well developed in Bhandara and Chandrapur

district. In these districts, calves diarrhea and neonatal mortality was reported, hence the present study was planned to analyze the circulating rotavirus infection by characterizing them by conventional and molecular approaches.

Detection of *Rotaviruses* by Latex agglutination test

In the present study, the ninety two (92) fecal samples from diarrheic and nondiarheic calves were tested by Latex agglutination test showed that 10 (10.86%) samples were positive for *Rotaviruses*. In Bhandara district total six (13.63%) fecal samples were positive for rotavirus infection, out of forty four(44) fecal samples from buffalo calves, 04 (18.18%) fecal samples and 02 (9.09%) from cow calves were positive. In Chandrapur district samples, out of 48 samples, 04 (8.33%) samples were positive for rotavirus infection by LAT from buffalo calves only. The present study indicated the buffalo calves were more affected in both the districts. The representative positive samples and negative for LAT is depicted in figure 1. Other researchers also reported that out of 53 diarrheic fecal samples screened for rotavirus infection in buffalo calves and cow calves, the LAT showed 17(32.08%) samples were positive (Singh and Jhala 2011). The current findings resemble with another study conducted in Maharashtra reported that out of 58 bovine calf fecal samples screened for rotavirus infections only 4 samples (6.89%) were positive by rapid rotavirus antigen detection kits (Tumlam *et al.*, 2018), and also in another study conducted in Amravati region of Maharashtra reported that out of 288 samples 35 samples were positive for rotavirus infection by LAT (Ade *et al.*, 2019). The bovine rotaviruses infection was variable in degree of incidence reported with various study by researchers with factor like geophilic area, climatic conditions, species

of animals, sex of animal, season of calving, managemental practices. The LAT is feasible for primarily screening, cost of kit is less and easy to perform.

Detection of *Rotaviruses* by Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

Detection of bovine *Rotaviruses* by RT-PCR with group specific VP6 gene

The ten (10) LAT positive samples were analyzed by molecular approach using group specific VP6 reverse transcriptase polymerase chain reaction (RT-PCR). The VP6 gene based RT-PCR yielded a specific product 309 bp in 03 samples from Bhandara districts and one samples from Chandrapur district figures 2. In another study Suresh *et al.*, (2011) reported that 27 samples were positive out of 112 by VP6 gene based RT-PCR, similarly another study Tumlam *et al.*, (2018) showed that four samples were positive for VP6 gene by RT-PCR out of fifty eight fecal samples while Basera *et al.*, (2010) studied that 10 samples were positive by group specific VP6 gene out of thirteen positive RNA-PAGE sample out of screened 128 diarrheic fecal samples from bovine calves. In another similar studies Ved *et al.*, (2016) reported that amplification of group specific VP6 gene based RT-PCR showed 50/157 cattle samples and 29/61 buffalo samples were positive.

From these studies it was observed that RNA PAGE, LAT, do not corroborate with RT-PCR detection owing to inhibitors in fecal sample for RT-PCR, or due to stages of disease and duration of sample collection for testing. In the present study LAT positive samples were analyzed however it was interesting to note that only buffalo calves samples were found positives, due to smaller sample size we can conclude that as buffaloes calf might be more severely affected or susceptible over cow calf.

Table.1 Primers used for G and P typing of Bovine Rotaviruses.

Primer	Sequence 5'-3'	Position	Amplicon size(bp)	Reference
G3	CGT TTG AAG AAG TTG CAA CAG	689~709	373	Gouvea <i>et al.</i> ,(1990)
G5	GAC GTA ACA ACG AGT ACA TG	760~779	302	Gouvea <i>et al.</i> ,(1994)
G6	GAT TCT ACA CAG GAA CTA G	481~499	581	
G8	GTC ACA CCA TTT GTA AAT TC	178~197	884	Gouvea <i>et al.</i> ,(1990)
G10	ATG TCA GAC TAC ARA TAC TGG	666~686	396	Iturriza-Gomara <i>et al.</i> , (2004).
P[1]	TTA AAT TCA TCT CTT AGT TCT C	1526~1505	460	Isegawa <i>et al.</i> , (1993)
P[5]	GGC CGC ATC GGA TAA AGA GTC C	1725~1704	659	
P[11]	TGC CTC ATA ATA TTG TTG GTC T	1398~1377	332	



Figure 1. Latex Agglutination test for group A rotavirus. LK08a-Positive Control, LK08b-Negative control. BAN42a-Positive Sample, BAN42b-Negative control. BAN43a-Negative sample, BAN-43b-Negative control.

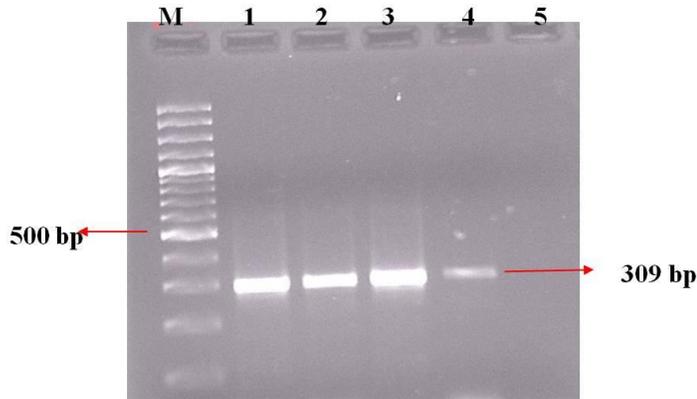


Figure 2: Agarose gel electrophoresis confirming amplicons of rotavirus by VP6 gene RT-PCR.

Lane M: 100bp Marker (Cat No.SM0321, Thermo Scientific, India)

Lane 1: BAN-42 Positive sample

Lane2: BAN-33 Positive sample

Lane3: BAN-31 Positive sample

Lane4: CAN-19 Positive sample

Lane5: Negative control

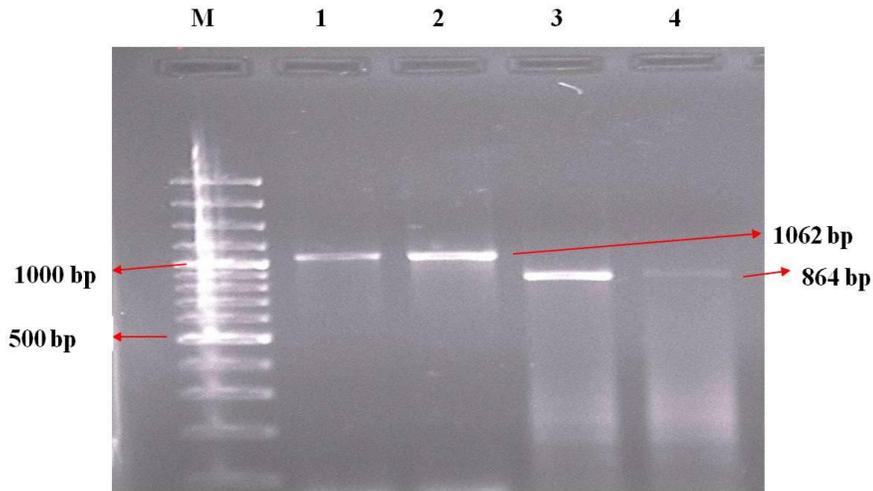


Figure 3 . Agarose gel electrophoresis confirming the amplicons of Rotavirus by VP7 and VP4 gene RT-PCR

M-Marker 100 bp (Cat No.SM0321, Thermo Scientific, India)

Lane-1: B 42 sample Bhandara positive for VP7

Lane-12-B31 sample Bhandara positive for VP7

Lane-13-B42 sample Bhandara positive for VP4

Lane-14-B31 sample Bhandara positive for VP4

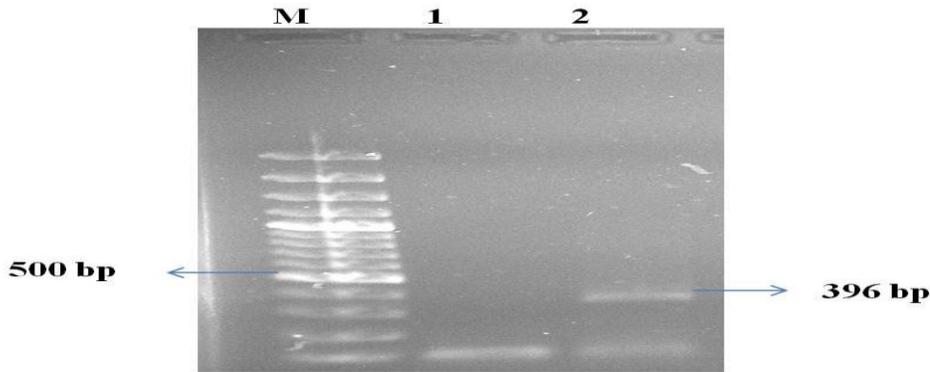


Figure 4 : Agarose gel electrophoresis showing amplicons of G-typing PCR

Lane M: Marker100bp (Cat No.SM0321, Thermo Scientific, India)

Lane 1: B-42 -Untype

Lane 2: B-31-G10 type

Detection of bovine *Rotaviruses* by RT-PCR with VP7 and VP4 gene

The LAT positive ten samples were further processed for VP7 gene and VP4 RT-PCR, it was observed that out of ten samples only two same B31 and B42 samples were positive for specific amplification 1062 bp for VP7 gene and 864 for VP4 gene. Both these fecal samples were from Bhandara districts as shown in figure 3. None of the samples from the Chandrapur districts were amplified by both (VP7 and VP4) gene specific RT-PCR. The researcher Chandrasekhar *et al.*, (2013) also reported that 25 positive samples from out of 184 fecal samples by full length VP7 gene. The Kashikar *et al.*, (2018) reported that three (3) samples, out of six positive samples were positive by RNA-PAGE in buffalo calves diarrhea were successful amplified with specific amplicon size. Current results were also agreement with the Sravani *et al.*, (2014) who reported that 17 samples were amplified for VP7 gene and four samples were amplified for VP4 gene out of 120 fecal samples were screened for RT-PCR. The researcher Ade *et al.*, (2019) reported that only one fecal sample was positive for amplification of VP4 gene out of 35 LAT

positive samples might be due to the presence of inhibitory substances in the fecal samples. The researcher Mukhtar *et al.*, (2016) reported that five fecal samples showed the specific amplification for VP4 gene by RT-PCR out of 12 ELISA positive samples obtained from screening of 200 fecal samples. The present study delineated that the PCR positivity is varied might be due to inhibitory factors or mismatched with 3' end of primer binding sites in Indian rotaviral strains.

G and P genotyping

Two samples were analyzed for G and P types in this study, one samples revealed specific amplified product for 396 bp as shown in figure 4, indicating G10 strain and one samples was untypeble, while none of the samples were shown specified amplification for P type namely P[1], P[5] and P[11]. It was observed that G10 genotype was predominant in past several studies, Gulati *et al.*, (1999) reported a frequency of G10 genotypes 83% isolated in Haryana and U.P, similarly, Wani *et al.*, (2004) reported that six out of ten sample were G10 type. In Madhya Pradesh (M.P.), central province of India Chandrasekhar *et al.*, (2013) studied that

circulation of divergent strains of G10 genotypes. The present study findings is contraindicated to the another study carried out by Niture *et al.*, (2011) where he reported that six samples shown positive amplification for VP7 gene specific amplification but none of the samples revealed to G6, G8 and G10 type specific products but two samples amplified for P[11] from six positive amplification VP4 gene. The researcher Beg *et al.*, (2010) reported that 25 samples (80.64%) revealed amplified products to G10 while three samples possessed G8 genotype, two sample were not amplified for by any G typing specific primer and none of the samples was amplified for G5 strain and while 31 samples revealed for P [11] amplification and none of the samples amplified for P [1] and [P5].

In conclusion the overall studies revealed that rotavirus infection can be detected by LAT in bovine fecal sample in a short time. It can be used on field test performance on farmer's doorstep, useful for early diagnosis of rotaviral diarrhea in calves and to avoid further economical losses in livestock by giving supportive therapy. The buffalo calves showed more positivity to rotaviruses infection than cow calves, however it required large data to conclude the susceptibility and severity of disease in these calves. However for rotavirus confirmation more than one test should be performed to avoid the false positivity/ negativity due to variation of amplification observed and LAT result correlation, it required further more data to conclusive remarks. The G typing indicated the G10 type in Bhandara districts however further typing by sequencing to further validates these isolate. Therefore molecular epidemiology needs to be carried out.

Acknowledgement

The authors are thankful to the Dean, Nagpur Veterinary College, Nagpur for providing

facilities and also to Indian council of Medical Research (ICMR), New Delhi (Sanction letter No.ZON/23/1/2017-ECD-II) for projects funds to the institute.

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How to cite this article:

Kadam, A. S., P. A. Tembhurne, S. W. Bonde, S. P. Chaudhary, N. V. Kurkure and Ingle, V. C. 2019. Molecular Detection of Group A Rotaviruses from the Bhandara and Chandrapur District of Maharashtra State, India. *Int.J.Curr.Microbiol.App.Sci*. 8(09): 2555- 2564.
doi: <https://doi.org/10.20546/ijcmas.2019.809.296>