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Molecular Characterization and Phylogenetic Relationship of VP6 Gene of Rotavirus among Canine, Porcine and Human Infants

Viraj Koul[1], S.R. Warke[1], U.M Tumlam[2], Mehak Tikoo[1], S.S. Bobade[3]

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ABSTRACT

Background: Rotaviruses have been widely reported and have a worldwide prevalence to be associated with diarrhea in humans but fewer studies abound on other mammalian species. Group A Rotavirus (RVA) are majorly responsible for causing acute gastroenteritis (AGE) in humans and animals.

Methods: In the present study 150 diarrheic fecal samples from, piglets, pups and human infants (0-5 yr of age) were screened for the presence of rotaviruses by lateral flow assay, VP6-based RT-PCR assay and phylogenetic analysis.

Result: A total of 13/150 (8.6%) fecal samples were found positive by lateral flow assay. Out of 50 diarrheic fecal samples each of human infants 4 (8.0%), pups, 03 (6.0%), piglet samples 6 (12.00%) showed the presence of rotavirus. All 13 positive fecal samples from piglets, pups and human infants were further screened for the detection of group A Rotavirus by VP6 gene-based RT-PCR assay. Of the samples tested, 13 (100.00%) samples were found positive for Rotavirus. The sequences of VP6 gene amplified from piglet, pups and human infants were subjected to phylogenetic analysis. The phylogenetic analysis showed that there is not much sequence variation between rotavirus from human infant and piglet. It showed more identity with *Homo sapiens* and *Bos taurus* which indicated the possibility of zoonotic transmission.

Key words: Canine, Human, Phylogenetic analysis, Porcine, *Reoviridae*, RV, VP6.

INTRODUCTION

Rotavirus is a notable enteric pathogen belonging to the *Reoviridae* family and responsible for causing acute gastroenteritis in humans and various species of animals including humans, pigs, calves, horses and dogs. They are icosahedral in shape, non-enveloped, possess either one, two- or three-layered capsid and are composed of 11 segments of double-stranded RNA (dsRNA) encoding 6 structural (VP1, VP2, VP3, VP4, VP5, VP6) and 6 non-structural proteins (NS1-NS6) (Suzuki, 2014).

The VP6 capsid protein is regarded as the group-specific inner capsid protein and is noted to have a high degree of nucleotide sequence conservation in differing viral strains (Kadam *et al.,* 2019). It also constitutes more than 50% of the capsid protein and is the major target of rotavirus detection by both serological and molecular methods (Mohan *et al*., 2014). So far, based on the genetic and antigenic differences nine different types of rotavirus have been identified denoted by the letters A -I. The rotavirus A species is the main source of infection in humans with species A-E being majorly responsible for infecting other animal species (Kirkwood, 2010). Zoonotic transmission of animal RVs, such as those from group A, to humans has been observed in the field and these animal RVs can cause diarrhea in humans (Alaoui *et al.,* 2020). Additionally, the segmented nature of the genome enables reassortment between/within human and animal strains and reassortment plays one of the major roles in the generation of the genomic diversity of this medically important virus (Komoto *et al.,* 2016). Until recently, specific rotavirus types have been associated with specific animal species. For example, human rotaviruses most commonly belong to G types 1-4 and P types [4] and [8]. Epidemiologically, there exists evidence for zoonotic transmission of rotaviruses. Human group A rotavirus strains that possess genes commonly found in animal rotaviruses have been isolated from infected children in both developed and developing countries (Geletu *et al.,* 2021). Currently,

[1]Department of Veterinary Microbiology and Animal Biotechnology T and R Cell, Nagpur Veterinary College, Nagpur-440 006, Maharashtra, India.
[2]Department of Veterinary Microbiology, Krantisinh Nana Patil College of Veterinary Science, Shirwal-412 801, Maharashtra, India.
[3]Department of Veterinary Public Health, Bombay Veterinary College, Mumbai-400 012, Maharashtra, India.

Corresponding Author: S.R. Warke, Department of Veterinary Microbiology and Animal Biotechnology T and R Cell, Nagpur Veterinary College, Nagpur-440 006, Maharashtra, India.
Email: shubhangi_warke@rediffmail.com

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36 G genotypes and 51 P genotypes have been identified in humans and animals worldwide (RCWG, 2018). G and P type combinations which are found in man have also been found in animal species. Some feline and canine rotavirus strains have spread into human populations as whole virions, bovine rotaviruses were involved in reassortment with human rotaviruses, leading to the emergence of unusual strains in various parts of the world (Geletu *et al.,* 2021). Various studies in India have also reported on the zoonotic potential of rotaviruses and detected various circulating genotypes (Tatte *et al.,* 2019; Rajendran and Kang, 2014). Studies on the molecular monitoring and probable zoonotic potential of rotaviruses among humans, pigs and dogs have not been extensively reported from the perspective of the Nagpur region of Maharashtra. In order to somewhat curtail the research gaps surrounding rotaviruses, which have become a global public health concern, the current study was carried out to determine the incidence of rotavirus in these chosen groups and to clarify any potential evidence of interspecies transmission and reassortment of rotaviruses among humans, pigs and canines.

MATERIALS AND METHODS

Collection of samples

The study was conducted in the Department of Veterinary Microbiology and Animal Biotechnology, T and R Cell, Nagpur Veterinary College from time-period of July 2021 to March 2022. Total of 150 diarrheic fecal samples were collected which comprised of pups (50) 0-1 yr., piglets (50) 0-6 months and human infants (50) 0-5 years. The date, time, location of sample collection, age, sex, breed, clinical signs, important clinical history and Vaccination status was recorded and the samples were transported to the laboratory in a container containing ice bag and stored at -20°C for further use.

Screening of fecal samples by lateral flow immunochromatography assay (LFA)

Each of the fecal samples was suspended in 10% phosphate-buffered saline (PBS, pH 7.2), clarified by centrifugation at 8000 × g for 10 min at 4°C and supernatants were collected and stored at -20°C till further use. The supernatant was used for determining the presence of rotavirus antigen by Lateral Flow Immunochromatography Assay (LFA). The processing of fecal samples was performed within 24 hours of collection of sample.

Fecal samples (50) of each group were screened by rapid Rotavirus Ag detection Ubioquick kit (Ubio biotechnology systems Pvt Ltd., Kerala, India) specific for canines, Rotachrom kit (Ubio biotechnology systems Pvt Ltd., Kerala, India) specific for porcine and Ubioquick kit (Ubio biotechnology systems Pvt Ltd., India) specific for humans respectively. The Interpretation of results was reported as positive when two lines, control (C) and test (T) appeared on the test strip. In case if the C line did not appear within 10 minutes, the test was considered non-valid and repeated with a fresh strip.

RT–PCR for VP6 gene of, piglets, puppies and human infants

RNA was extracted from fecal samples by Trizol method (Ambion, Life technologies, North America) and quantified using nanodrop (Thermoscientific, USA). cDNA synthesis was carried out using high-Capacity cDNA Reverse Transcription Kit (Promega) and stored at -20°C till further use. Polymerase chain reaction was used to amplify the VP6 gene of Rotavirus using the primers described by Falcone *et al.* (1999) (Table 1).

RT PCR to amplify VP6 gene

The Cyclic conditions in Master gradient cycler were carried out as: Initial denaturation at 94°C for 5 min, 35 cycles each of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. Amplicons were visualized for appropriate band size under a UV transilluminator and photographed in gel documentation unit.

Sequencing and phylogenetic analysis

The gel-eluted PCR products were sequenced from commercial sequencing services. The VP6 gene (03) raw sequences were first analyzed for the base call and trimmed using the Chromas software (version 2.5.1). The cleaned sequences were blast for homology search using the BLASTn interface of Gene Bank (http://blast.ncbi.nlm.nih.gov/). All sequences were first aligned by using Clustal W 1.6 software. Further, the aligned sequences were converted into MEGA file for the construction of a Phylogenetic tree using MEGA 10.0 software as per the method of Kumar *et al*. (2018) with 32 homologous sequences retrieved from GenBank (http://www.ncbi.nlm.nih. gov/genbank/index.html).

RESULTS AND DISCUSSION

Incidence of rotavirus in human infants, pups and piglets

A total of 150 diarrheic fecal samples suspected to be positive for rotavirus were screened by rapid lateral flow antigen detection test and 4 out of 50 human infants (8.0%), 3 out of 50 pups (6.0%) and from 6 out of 50 piglet samples (12.00%) were positive. Similar results were detected in case

Table 1: List of oligonucleotide Primers used for VP6 gene RT-PCR.

Gene	Primers sequence (5′-3′)	Amplicon size	Reference
VP6-F	5′GACGGVGCRACTACATGGT3′	379 bp	Falcone (1999)
VP6-R	5′GTCCAATTCATNCCTGGTGG3′		

of human infants, pups and piglets by Tumlam *et al.* (2018) revealing the incidence of rotavirus to be about (4.0%; 9.85%). Singh *et al.* (2017) reported 18.75% of rotavirus incidence in human infants which was in consensus with results obtained in our study. This suggests that the LAT test, albeit less sensitive, is useful for early detection of infected animals.

The incidence of rotavirus in different species is depicted in (Table 2) and (Fig 1).

Age wise incidence of rotavirus

The results of present study indicated that positivity of rotavirus in human infants was recorded as (2% and 6%) for the age group of 0-1 month and > 6 months respectively. The result is depicted in (Table 3). Our results revealed that the prevalence of rotavirus infection was higher in human infants of more than 6 months age group. These observations were similar to the earlier reports in which John *et al.* (2014) reported that the maximum number of patients were in the age group of 6 to 15 months. This could be due to the fact that the maternal antibodies may be protecting the infants against infections during early stage of life. The percent positivity in piglets was recorded as 2% in 0-1 month, 8% in 1-3 months and 2% in >6 months age groups respectively. Our findings showed more prevalence of rotavirus infection in piglets of 1-3 months age group similar to the results obtained by Tumlam *et al.* (2018) which reported that the incidence of rotavirus was higher in piglets of 1-3 months age group.

In pups, the percent positivity of (2% and 4%) were recorded in 3-6 month and >6 months age group respectively. Our findings revealed a higher prevalence of rotavirus infection in pups of more than 6 months age group. Similar findings were obtained by Tumlam *et al.* (2018) which revealed that the prevalence of rotavirus infection in pups was (1/17; 5.88%) in 3-6-month age group.

Table 2: Incidence of rotavirus in human infants, pups and piglets.

Species	Samples tested	Type of test used	Rotavirus +ve	Per cent positivity
Pups	50	Rapid *rota virus* Ag detection ubio kit	3	6.00%
Piglets	50	Rapid *rota virus* Ag detection rotachrom kit	6	12.00%
Human infants	50	Rapid *rota virus* Ag detection ubio kit	4	8.0%
Total	150		13	8.6%

Table 3: Age-wise incidence of rotavirus in human infants, pups and piglets.

Name of species	Type of test used	Age (in months)				
		0-1	1-3	3-6	>6	Total (positive/ total samples)
Pups	Rapid rota virus Ag detection ubio kit	-	-	1	2	3/50
Piglets	Rapid rota virus Ag detection rotachrom kit	1	4	1	-	6/50
Human infants	Rapid rota virus Ag detection ubio kit	1	-	-	3	4/50
		Total				13/150

C= Control line and T= Test line

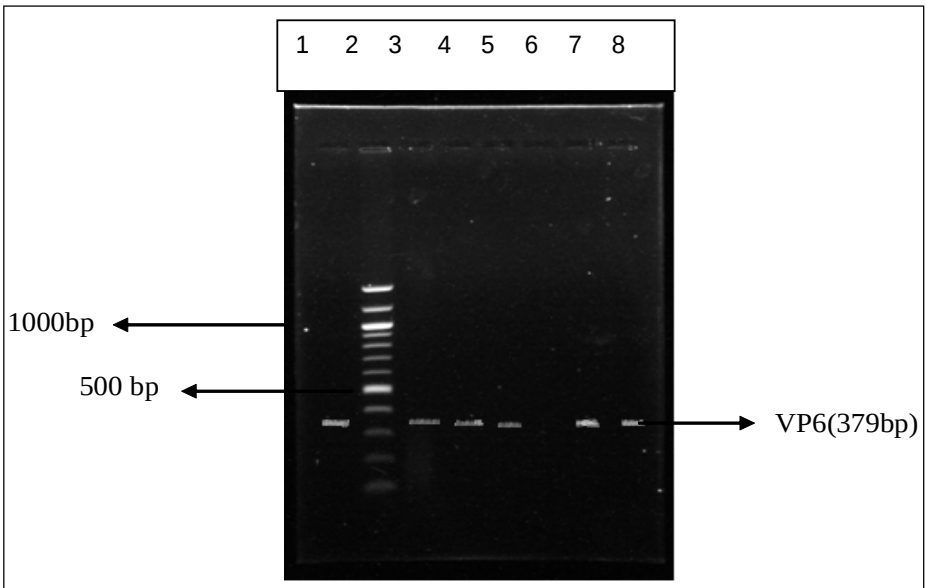
Fig 1: Positive lateral flow assay test for rotaviruses specific for humans (HRV-32 and 19), pigs (PRV-16 and 32) and canine (CRV-8 and 18).

Molecular characterization of rotavirus by Targeting of VP6 gene by RT-PCR

RT-PCR is a highly advantageous technique which has been employed previously as a specific and sensitive molecular assay for detection of rotavirus VP6 gene. In the present study already published primer of Falcone (1999) were used to amplify VP6 gene which yielded 379 bp amplicon size. A total of 13 (100.00%) fecal samples from piglets, pups and human infants were found positive for group A Rotavirus by VP6 gene-based RT–PCR assay. The result of Rotavirus detection by VP6 gene-based RT–PCR assay is as shown in Fig 2. Similar findings by Tumlam *et al.* (2018) revealed 43 out of 44 samples (97.72%) positive for rotavirus infection. Furthermore, samples from all species (bovine calves, piglets, kids, lambs and pups) were 100% positive for group A rotavirus by VP6 gene-based RT-PCR assay with the exception of human infants. Shams *et al.* (2020) detected (22/130; 16.9%) samples as positive for rotaviral gastroenteritis which were significantly lower than the results obtained in our study. The reason for it might be the presence of non-specific inhibitors in the fecal samples (Raorane *et al.,* 2020).

Phylogenetic analysis of VP6 gene from piglets, pups and human infants

The sequences of VP6 gene amplified from piglet (P-16), pups (C-8) and human infants (H-19) were subjected to phylogenetic analysis. As per BLAST search with BLASTN program against non-redundant database the sequence of VP6 gene isolated from human infant (H19) found to be showcasing high diversity and out-group from all the sequences analyzed and may be showing defined diversity among tested sequences. P-16 was found to be homologous (93.80 % homology) with other sequence obtained from pig of Maharashtra region (Accession No. LC379954.1). The sequences obtained from canine sample (C-8) was found to have 100% identity with a sequence from China (Accession No. OL388440.1). Although, the P-16, C-8 and H-19 sequences were distantly placed, they were closely related to the sequences obtained from China, Thailand and the USA, while, other sequences of human samples from China, Maharashtra (India), USA, Bangladesh, Japan were placed distantly (Fig 3). Similar results were obtained in a study by Chitambar *et al.* (2009) where it was revealed that VP6 gene of strain NIV929893 (obtained from 11-month-old infant) showed (92.3-92.8%) nucleotide and (98.2-99%) identities to human rotavirus strains. Findings by Tumlam *et al.* (2018) were in accordance with our study revealing that the VP6 gene of piglet rotavirus showed higher identity with humans, bovine from Cambodia and South Korea indicating the possibility of zoonotic transmission. Additionally, a study by Matthijnssens *et al.* (2008) reported that there was a common origin between human rotavirus strains and porcine strains. Yan *et al.* (2019) reported that the strain of rotavirus (RVA/Dog-tc/CHN/SCCD-A/2017/G9P[23]) obtained from dog was closely related to porcine RVA strains or porcine-like human RVA strains.



Lane 1= Positive control (Human vaccine); Lane 2= Molecular weight marker (100 bp ladder); Lane- 3,4 and 5= Human infants (H-9, H-32 and H-19); Lane-6= Negative control; Lane-7= Piglets (P-16); Lane-8= Pups(C-8).

Fig 2: Screening of VP6 gene of Rotavirus in fecal Samples by RT-PCR.

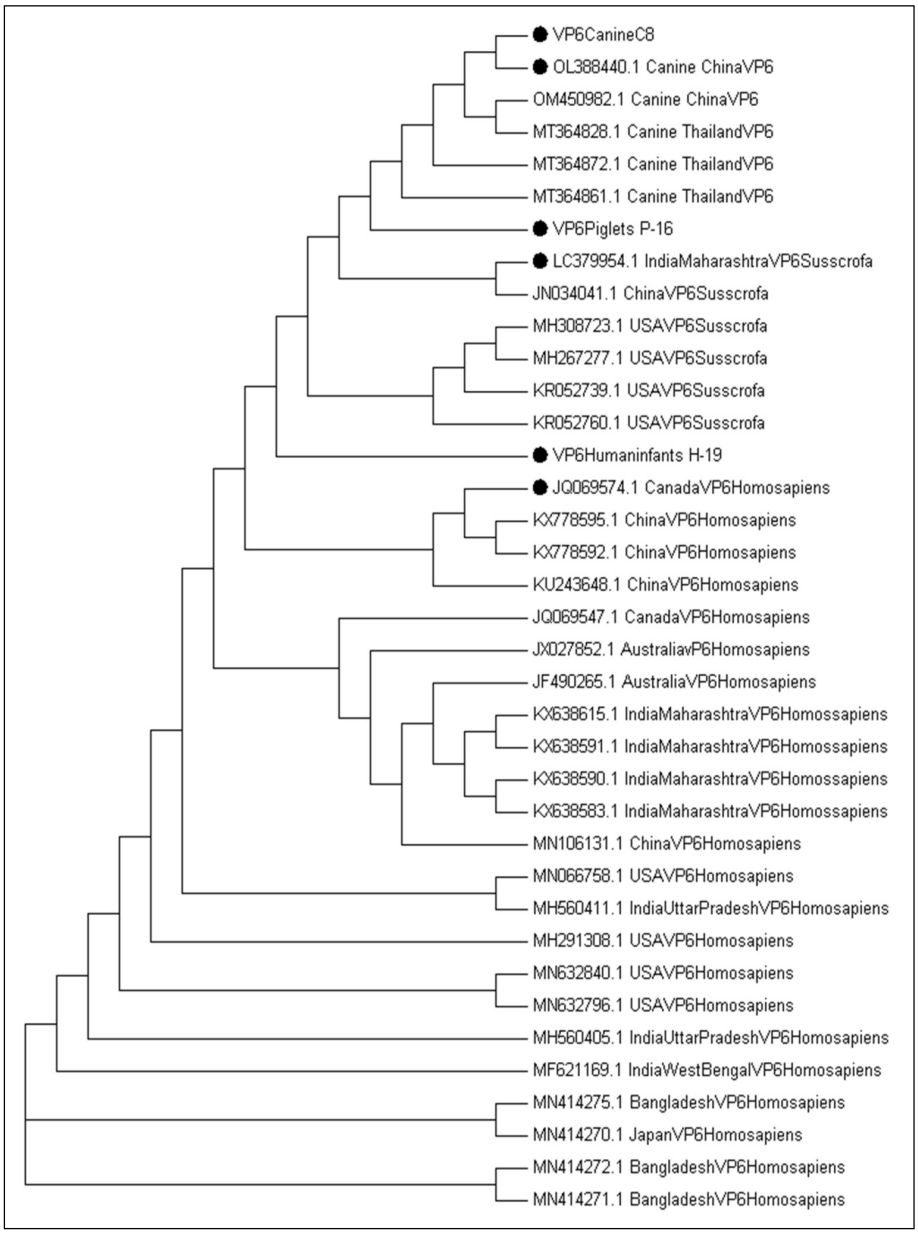


Fig 3: Phylogenetic tree of Partial gene sequence of canine, piglets and human infants of VP6 gene.

CONCLUSION

The advances in nucleotide sequence detection methods have enabled the detection and identification of Rotavirus genomes from various animals. The study of evolutionary history and genetic diversity provides basic knowledge to study the potential of zoonotic transmission of rotavirus. In our study, the phylogenetic analysis showed that there is not much sequence variation between rotavirus from human infants and piglets. It showed more identity with *Homo sapiens* and *Bos taurus* which indicated the possibility of zoonotic transmission. The rotavirus gene might be re-assorting in between the different species, the result showed the intense interspecies virus integrity in terms of nucleotide identity as well as phylogeny arrangement within the cluster. Rotavirus of different species evolving fast by exchanging the gene sequence from their surroundings. This is due to intensive integrated farming, increasing in human to animal interaction in day-to-day life. Our study suggests that there may be possibilities of future epidemic by heterologous rotavirus as it is evolving from interspecies interaction.

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Consent for publication

All the authors consent to the publication of this manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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