



Molecular Characterization and Phylogenetic Relationship of VP4 and VP7 Genes of Rotavirus Group A among Porcine, Canine and Human

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ABSTRACT

Background: Rotaviruses are major enteric pathogens that cause acute gastroenteritis infections in both young children and animals across the globe. Group A Rotavirus is the most prevalent group that affects both humans and animals and is further divided into various G and P types based on the antigenic and molecular characterization of the VP4 and VP7 genes. VP7 determines G genotype, whereas VP4 determines P genotype. A major factor in the evolution of rotaviruses, contributing to the diversity of viruses in humans and animals, is the inter-species transmission of RVAs.

Methods: A total of 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 test. The samples were further tested for rotavirus group A (RVA) by using one-step RT-PCR specific to the VP4 and VP7 genes. The genetic relationship between VP7 and VP4 partial gene sequences of human infants and piglets was evaluated using phylogenetic studies.

Result: In the present study, 13/150 (8.6%) fecal samples were found positive for rotavirus antigen by lateral flow test. Out of the diarrheic fecal samples, human infants (4/50; 8.0%), pups (03/50; 6.0%) and piglet samples (06/50; 12.00%) were positive for rotavirus. All the positive samples screened for the detection of group A Rotavirus by amplifying VP7 and VP4 genes using RT-PCR revealed that 1/6 (16.66%) and 3/4 (75%) were found positive from pigs and humans, respectively. VP4 partial sequences of H-19 and P-16 samples shared greater homology with the sequences of the VP4 gene retrieved from pigs in the USA than with those from human sequences of Maharashtra and other sequences from India. VP7 gene sequences of human infants (H-19) and piglets (P-16) were placed under different clusters. Implying the possible zoonotic relevance of this virus, the phylogenetic analysis of sequences retrieved from human newborns and piglets revealed the possibility of interspecies transmission of the rotaviral strain circulating in Nagpur.

Key words: Interspecies transmission, Phylogeny, Rotavirus, VP4, VP7.

INTRODUCTION

Rotaviruses are major enteric pathogens affecting humans and various animal species, including cattle, buffalo and pigs and they are responsible for causing acute gastroenteritis (Ferrari *et al.*, 2023). Transmission of rotavirus occurs via the faeco-oral route and manifests as symptoms of vomiting, diarrhoea, fever, abdominal cramps and dehydration (Jampanil *et al.*, 2023). Rotaviruses are categorized into 10 groups (A-J) based on their genetic variability (Yandle *et al.*, 2020). Group A rotaviruses (RVA) are spread worldwide and divided into G and P types based on their antigenic characteristics. The G genotype is determined by VP7 (glycoprotein), while the P genotype is determined by VP4 (protease-sensitive protein) (Wengui *et al.*, 2023). The spike protein VP4 and the outer protein VP7 of rotavirus can elicit neutralizing antibodies, making them potential targets for vaccine development (Asensio-Cob *et al.*, 2023). To date, strains from humans and animals worldwide have been characterized with 36 G genotypes and 51 P genotypes (Liu *et al.*, 2021). Rotavirus's genomic diversity and ability to infect multiple animals indicate the potential for highly pathogenic variations. Gene reassortment events can lead to the spread of zoonotic illness to humans (Bennett *et al.*, 2021).

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Previously, documented studies have revealed that pig and human RVA strains have undergone genetic reassortment, sharing multiple gene segments, suggesting potential interspecies transmission (Ndebe *et al.*, 2023). Since the information on prevalence and genetic diversity of rotavirus in Nagpur is scarce. Therefore, continuous surveillance is

necessary for comprehending the epidemiology of rotaviruses. The present study aimed to investigate the prevalence and molecular features of rotaviruses in clinically diseased animals and humans of Nagpur.

MATERIALS AND METHODS

Collection and processing of faecal samples

The research was conducted in the Department of Veterinary Microbiology, Nagpur Veterinary College, Nagpur during year 2021-2023. 150 diarrheic fecal samples from pups (50) 0-1 yr, piglets (50), 0-6 months and human infants (50) 0-5 yrs were collected from diarrheic animals from July 2022 to March 2023. The samples were transported to the laboratory in a container containing an ice bag and stored at -20°C for further use. Each of the faecal 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.

Screening of faecal samples by lateral flow immunoassay

Screening of faecal samples of pups (50), piglets (50) and human infants (50) for the presence of rotavirus was carried out by commercially available rapid Rotavirus Antigen detection (Ubio and Rotachrom kits, Ubio biotechnology systems Pvt Ltd., Kerala, India).

RNA extraction and cDNA synthesis

RNA was extracted by the Trizol (Ambion, Life Technologies, North America) method with slight modifications (Bhosle *et al.*, 2023) and quantified using a nanodrop. cDNA synthesis was carried out using a cDNA synthesis kit from high-capacity cDNA Reverse Transcription Kits of Promega, USA. The primer sequences utilized to amplify the VP4 and VP7 genes of rotavirus are depicted in (Table 1).

RT-PCR thermocycling conditions for VP4 gene

A 50 µl reaction volume was set up comprising of 10.0 µl of 5X PCR buffer, 1.0 µl of 25 mM MgCl₂, 1.0 µl of 25mM dNTP, 2.0 µl of 10 pmol each Con3 Forward and Con2 reverse primers, 0.3 ml of Taq DNA polymerase adjusted with 31.7 µl of DEPC water. 2.0 µl of cDNA synthesized from 500 ng of

total RNA was added to this reaction mixture. The cyclic conditions were carried out as: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 2 min, extension at 72°C for 3 min, followed by final extension at 72°C for 10 min and hold at 4°C.

RT-PCR thermocycling conditions for VP7 gene

A 50 µl reaction volume was set up comprising of 10.0 µl of 5X PCR buffer, 1.0 µl of 25 mM MgCl₂, 1.0 µl of 25mM dNTP, 2.0 µl of 10 pmol each BEG9 Forward and END9 reverse primers, 0.3 µl of Taq DNA polymerase adjusted with 31.7 µl of DEPC water. 2.0 µl of cDNA synthesized from 500 ng of total RNA was added to this reaction mixture. The cyclic conditions were carried out as: Initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 46°C for 2 min, extension at 72°C for 2 min, followed by final extension at 72°C for 10 min and hold at 4°C.

Sequencing and phylogenetic analysis

PCR amplicons for VP4 and VP7 genes were sequenced from the commercial sequencing services (M/S. Eurofins Genomics India Pvt. Ltd., Bangalore). The raw sequences obtained after commercial sequencing were first checked for base call and trimmed using the Chromas software (version 2.5.1). The cleaned sequences were BLAST for the homology search using BLASTn interface of the Gene Bank and sequences of target genes; The global sequences for VP4 and VP7 were retrieved. VP4 and VP7 gene sequences of the present study were analyzed pairwise and multiple sequence alignment was performed using the ClustalW embedded in the MEGA XI software (<http://www.megasoftware.net/>). Using the neighbor-joining method, the phylogenetic tree was constructed based on the Maximum composite likelihood model. Furthermore, homology between the amino acid sequences of VP4 and VP7 genes was ascertained by a dot plot matrix generated by the EMBOSS dot matcher tool.

Dot plot matrix for translated amino acids

A dot plot matrix for amino acid similarity was created to study the similarity of the amino acids. Two different amino acid sequences were compared by plotting their residues along a matrix, where identical residues were represented

Table 1: List of oligonucleotide primers.

Name of primer	Sequence of primer for VP4 gene amplification	Total nucleotides	Amplification size	Reference
First round PCR primers				
Con3 11-32	5'TGG CTT CGC TCA TTT ATAGACA 3'	22	876 bp	Gentsch <i>et al.</i> (2005)
Con2 868-887	5' ATT TCG GAC CAT TTATAA CC 3'	20		
Sequence of primer for VP7 gene amplification				
First-round PCR primers				
BEG9 1-28	5'GGCTTTAAAGAGAGAATTTCCGTCTGG3'	28	1062 bp	Gouvea <i>et al.</i> (1990)
END9 1062-1036	5'GGTCACATCATACAATTCTAATCTAATCTAAG3'	31		

by dots. This often helps visualize regions of similarity and difference Batzoglou (2005).

RESULTS AND DISCUSSION

Detection of rotavirus by lateral flow assay

A total of 13/150 (8.6%) faecal samples were found positive. From 50 diarrheic fecal samples of human infants, 4 (8.0%) were found positive, while out of 50 samples of pups, 03 (6.0%) were found positive and from 50 piglet samples, 6 (12.00%) showed the presence of rotavirus (Fig 1; Table 2). Studies by Tumlam *et al.* (2018), Biswas *et al.* (2019) and Pawar and Karande (2022) all showed greater rotavirus prevalences in human infants (22%, 18.75%, 36%, 63.3% and 28.81%), which was in contrast to our findings. (Tumlam *et al.*, 2018) revealed the prevalence of rotavirus in pups and piglets to be 4.0% and 9.85%. LFA tests are advantageous in early detection of rotaviruses owing to rapid, sensitive and specific results.

Age-wise distribution of rotavirus

Out of 50 human infant stool samples tested, 04 (8.00%) were found to be positive for rotavirus infection. The results of this study indicated a prevalence of 2% and 6% for the age groups of 0-1 month and > 6 months, respectively. Our results demonstrated that the prevalence of rotavirus infection was higher in human infants of the greater than 6

months age group. These findings aligned with the earlier reports by Pawar and Karande (2022) which reported that the maximum number of infected children were in the age group of 7 to 12 months. The primary cause of severe diarrhoea in children in both industrialized and poor nations is rotavirus. By the age of five, practically every child has contracted the infection (Singh *et al.*, 2017).

Out of 50 piglet fecal samples screened, 06 (12.00%) were positive. The percent positivity of (2%, 8%, 2%) was reported as 0-1 month, 1-3 months and >6 months age group, respectively. Our findings showed a higher prevalence of rotavirus infection in piglets of the 1-3-month age group. (Tumlam *et al.*, 2018) reported that the prevalence of rotavirus was higher in piglets of the 1-3-month age group. Findings by (Wu *et al.*, 2022) were in contrast to our findings and reported a higher prevalence of rotavirus infection in piglets less than 1 month age. Presence of maternal antibodies can be the reason for lower prevalence in >1 month old piglets.

In the present study, out of 50 fecal samples screened, 3 (6.00%) were positive for rotavirus in pups. The percent positivity of (2% and 4%) was reported in 3-6 months and > 6 months age groups, respectively. Our findings reported a higher prevalence of rotavirus infection in pups of the more than 6-month age group. Tumlam *et al.* (2018) reported the prevalence of rotavirus infection (1/17; 5.88%) in the 3-6-month age group of pups. The reason for the development of infection in this age group of piglets and pups could be due to the underdeveloped immune system.

Molecular characterization of rotavirus amplifying VP7 and VP4 genes by RT-PCR

Piglets

All the samples positive by lateral flow of piglets were subjected to RT-PCR of VP7 and VP4 genes, which yielded amplicons of 1062 and 876 bp, respectively. Our findings revealed that (1/6, 16.66%) of each preliminary positive sample tested were found positive for VP4 and VP7 gene-based RT-PCR, respectively (Fig 2; Fig 3; Table 2). Similar results were found in a study by (Malik *et al.*, 2013), which revealed the overall prevalence of rotavirus infection in swine to be 13.04%. Moreover, a study by Kylla *et al.* (2018) revealed that the overall prevalence of rotavirus based on targeting the VP7 gene in RT-PCR was (7.43%; 34/457). Pegu *et al.* (2017) documented that 14 (13.1%) out of 107 samples were positive for rotavirus in piglets by RT-PCR. Vaccinating piglets and gilts against rotavirus is the most effective way, as gilts produce antibodies. Management practices, low humidity, high population density and primiparous female density can all enhance rotavirus prevalence in a herd (Camargo *et al.*, 2012).

Human infants

In the present study, RT-PCR of the VP7 and VP4 gene yielded, amplicon of 1062 and 876bp (Fig 2; Fig 3; Table 2). Our results revealed that (3/4, 75%) of each preliminary positive sample tested were found positive for rotavirus

Table 2: Prevalence of rotavirus in fecal samples of different species.

Species	Rotavirus +ve by lateral flow	Amplification of VP4 gene RT-PCR	Amplification of VP7 gene
Pups	3	00	00
Piglets	6	01	01
Human infants	4	03	03
Total	13	04	04

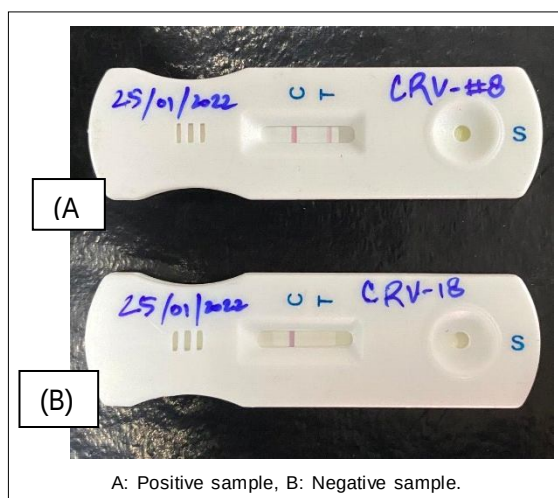


Fig 1: Ubioquick VET rotavirus antigen rapid test kit depicting positive and negative reaction for rotavirus antigen.

infection in human infants by VP4 and VP7 gene-based RT-PCR, respectively. Arun *et al.* (2019) revealed that all the positive samples, when tested by RT-PCR targeting the VP7 and VP4 genes, amplified to give an expected amplicon size of 1062 bp of the VP7 gene and 876 bp of VP4 gene. In another study by Alkali *et al.* (2016), it was revealed that (23/38; 60.5% and 27/38; 71.1%) samples obtained from diarrheic children in Sokoto, Nigeria was positive for VP4 and VP7 based RT-PCR, respectively. Rotavirus is a significant cause of infection and hospitalization in children under 5 years old, possibly due to early environmental exposure and undeveloped immune

systems increasing their susceptibility to diarrhoea (Njifon *et al.*, 2024).

Pups

All the samples of pups positive by lateral flow assay were further subjected to RT-PCR of VP7 and VP4 gene, but it failed to yield a positive amplicon (Table 2). The reason for these variable findings was detailed in studies by Deswal *et al.* (2015) and Gill *et al.* (2017) suggested that the existing non-specific PCR inhibitory substances in the fecal samples and the non-specific primary binding sites may be the cause of non-amplification of the desired gene.

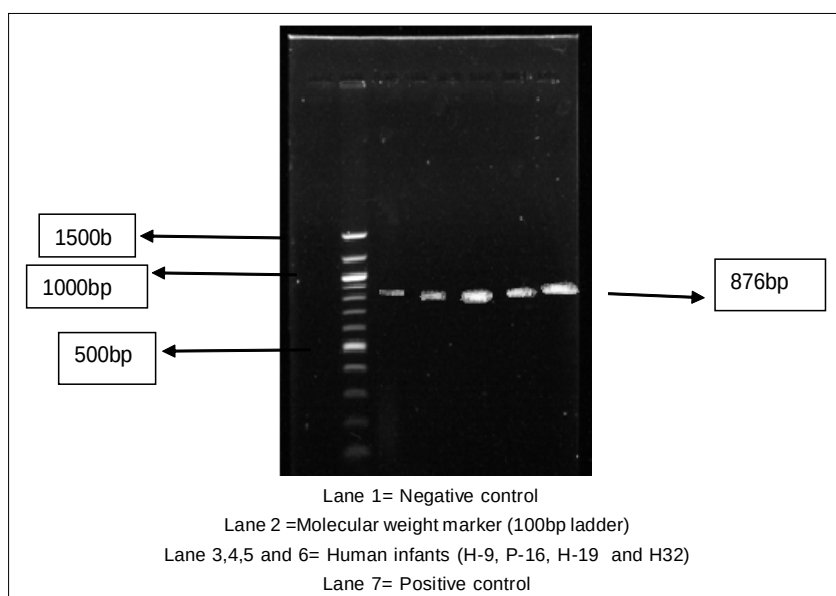


Fig 2: Screening of VP4 gene of Rotavirus in faecal samples by RT-PCR.

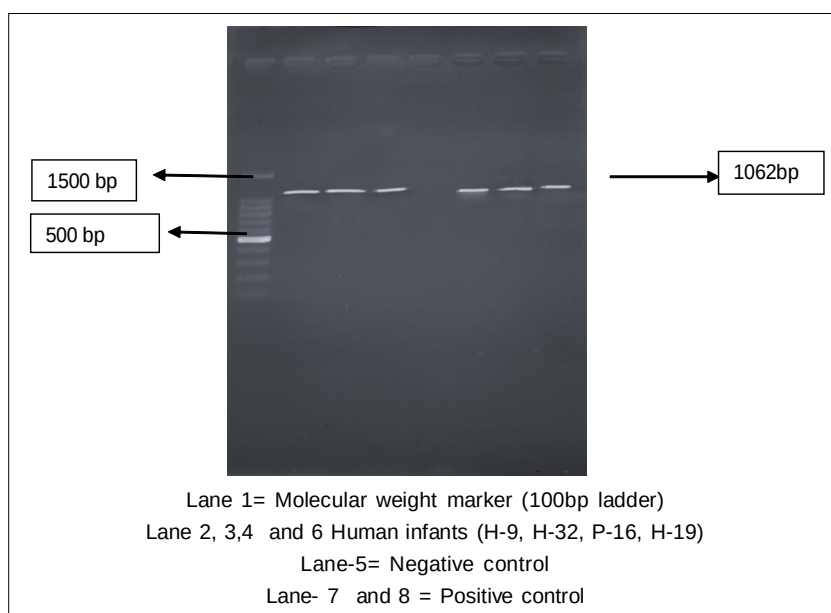


Fig 3: Screening of VP7 gene of Rotavirus in faecal samples by RT-PCR.

These results may be related to the sequence variations detected in the circulating Indian rotavirus strains and novel methods such as adapter ligation amplification could relieve this problem.

Phylogenetic analysis of VP4 gene of piglets and human infants

The phylogenetic analysis of VP4 gene sequence, amplified from a human infant (H-19) fecal sample, was placed under the same cluster as that of the DNA sequence obtained from pig (P-16) (Fig 4). The amplified sequences showed interspecies cross-relation with more than 90% homology. This implies that the VP4 gene sequences of the rotavirus amplified from pigs (P-16) and human infants (H-19) in the Nagpur region do not differ significantly.

The sequence of VP4 obtained from H-19 was found to be closely related (97.47 % homology) to a sequence of human sample from Maharashtra (Accession No

LC377479.1). Similarly, the sequence of P-16 was found to be closely related (93.80% homology) to a sequence obtained from pig (Accession No LC377486) from Maharashtra. The sequences of both H-19 and P-16 shared greater similarity with the sequences of VP4 gene amplified from pigs from USA (Accession Nos. KX527790, KX527800 and KX527801) and those from human sequences of Maharashtra (Accession Nos. KX646610, KX646589, MK039112, MK043941) and other sequences from India (Fig 4). The human and porcine strains were further distantly related to a few human strains from Japan and the USA. Animal rotaviruses may be the source of human infection, with genetic sequences showing close similarities. Novel swine strains infect humans due to rotavirus evolution in pigs (Kylla *et al.*, 2018). A study by Tumlam *et al.* (2018) revealed that the VP4 gene of bovines (C-41) and pigs (P-371) showed homology to those of humans (H-70) and bovines. Furthermore, Camargo *et al.* (2012)

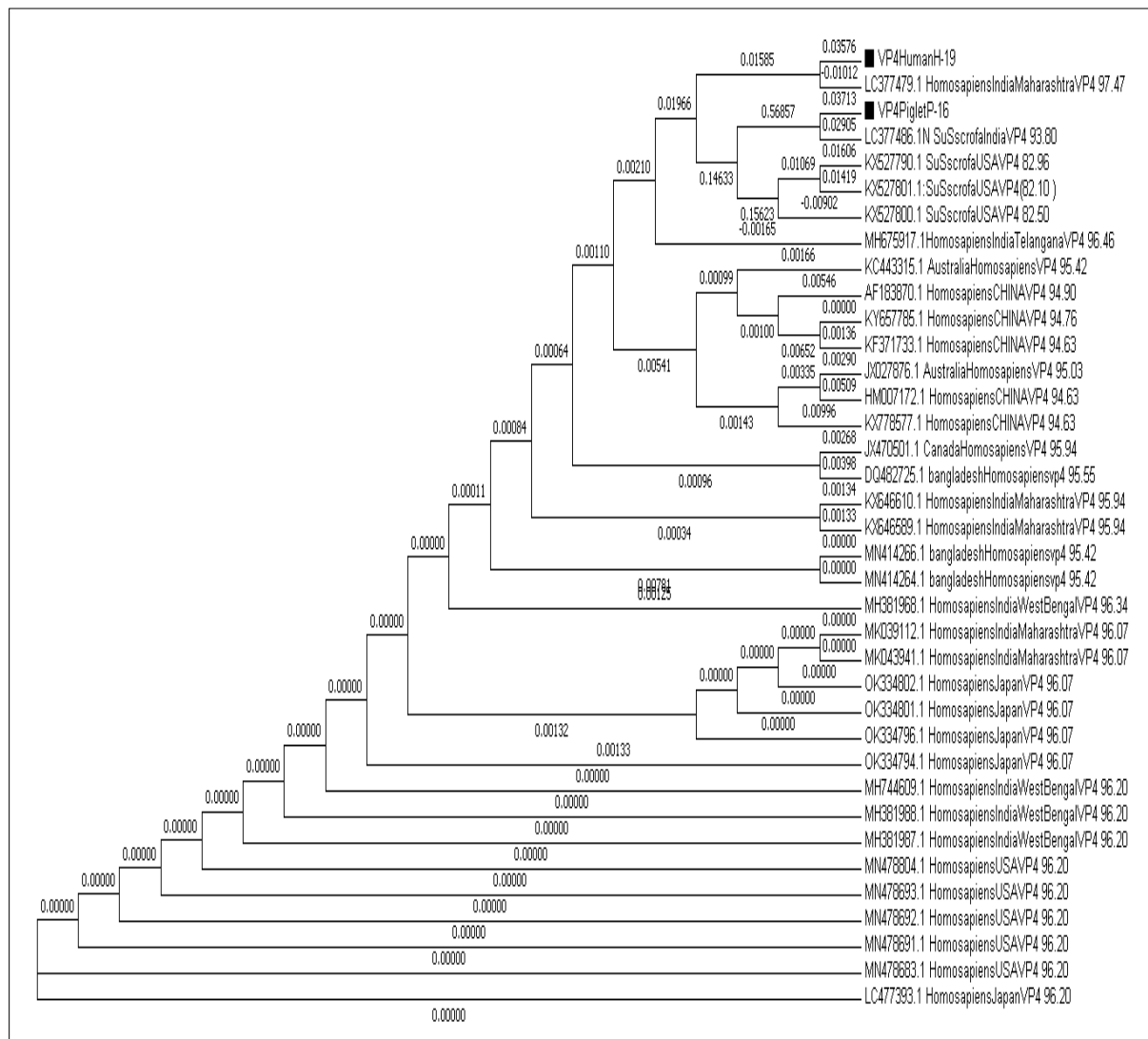


Fig 4: Phylogenetic tree of the partial gene sequence of the VP4 gene of piglets and human infants.

reported that the VP4 gene was identified as P [23] genotype in four positive samples (4/17; 23.5%) with amino acid sequences closely related (95.9-96.5%) to the Hokkaido-14 strain (Japan), also found in pigs. Earlier studies have reported that human and porcine rotaviruses frequently cross species boundaries and recombine, highlighting the dynamic interaction between these two species (Qiao *et al.*, 2024).

Phylogenetic analysis of VP7 gene of piglets and human infants

Phylogenetic tree of VP7 gene sequences from human infant fecal sample (H-19) and piglet sample (P-16) revealed that they were remotely located from each other and were placed under distinct clusters (Fig 5). VP7 sequence of H-19 was closely related (97.33% homology) to that of the sequence from the human sample of Maharashtra (Accession no. LC377480.1). VP7 sequence obtained from P-16 was found to be closely related (100 % homology) to the sequence of a pig sample from Maharashtra (Accession No LC377485.1). H-19 sequence had more homology with the sequences obtained from humans of India and Japan. Likewise, the sequence of P-16 showed more homology with the sequences from India, USA and

Japan. A few other human VP7 sequences from Maharashtra, Japan, China and the USA were distantly located from the VP7 sequences obtained in the present study (Fig 5). Transmission of G4 and P[6] genotypes from pigs to humans in China was noted and the genotypes exhibited strong similarity to those of porcine strains (Mao *et al.*, 2022). These findings differed from those obtained in our investigation, which found that the VP7 sequences of H-19 and P-19 were clustered separately.

Dot plot matrix for translated amino acids

The dot plot matrix of the partial amino acid sequence of the VP4 gene shows a strong match of pig (P-16) sample from 20-85 amino acids with the human from 5-75 amino acids (Fig 6). Similarly, the dot plot matrix of the partial amino acid sequence of the VP7 gene showed match of pig (P-16) sample from 1-140 amino acids with the human from 60-200 amino acids (Fig 7). A recent study reported that the VP4 gene sequence of the porcine isolate encoded 777 amino acids and showed high similarity (99.8-99.9%) with an RVA isolate obtained from a pig in China (Lv *et al.*, 2024). Similar results were noted in the case of VP7 gene sequence showing 94.3-96.6% homology to the porcine and human sequences from China. The homology analysis

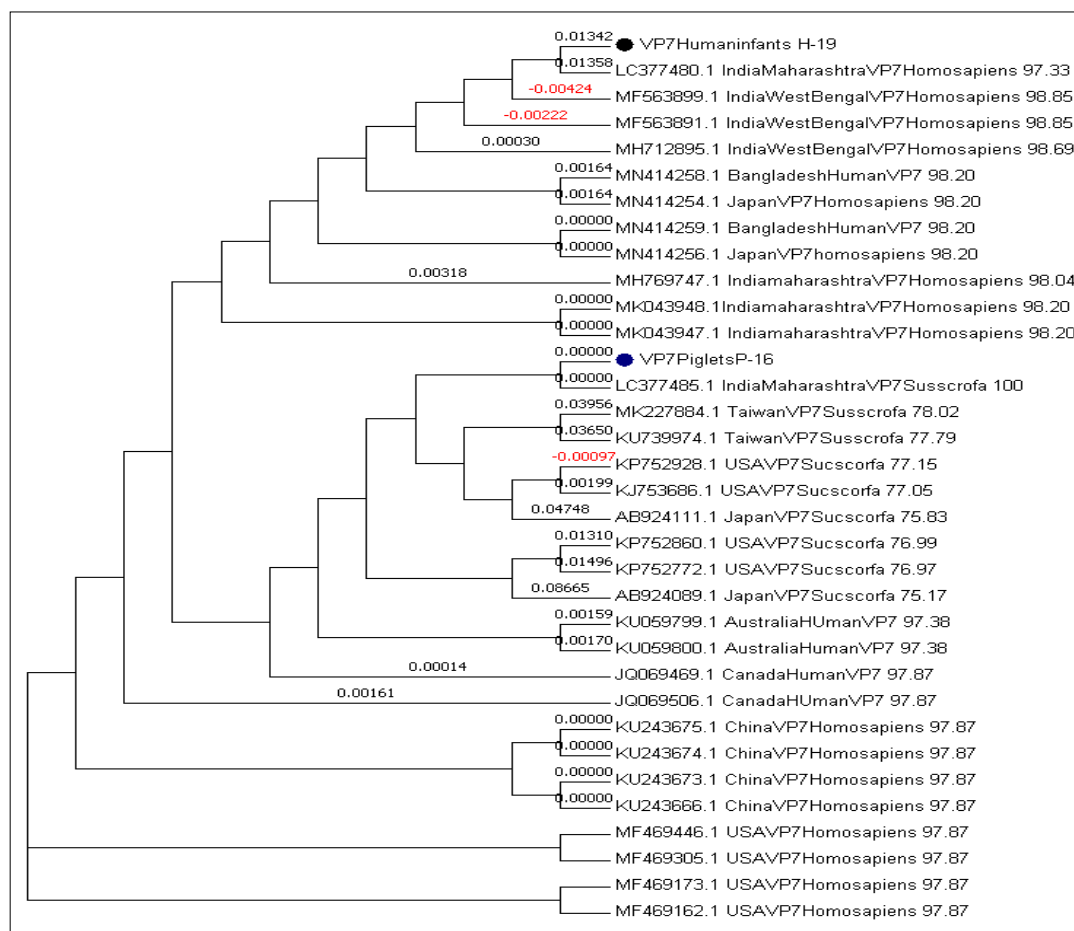


Fig 5: Phylogenetic tree of partial gene sequence of the VP7 gene of piglets and human infants.

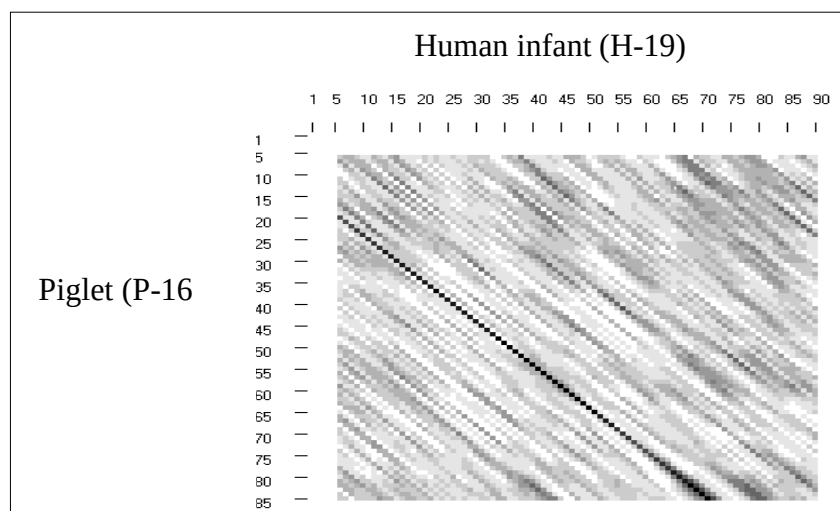


Fig 6: Dot plot matrix for translated amino acid of the VP4 gene.

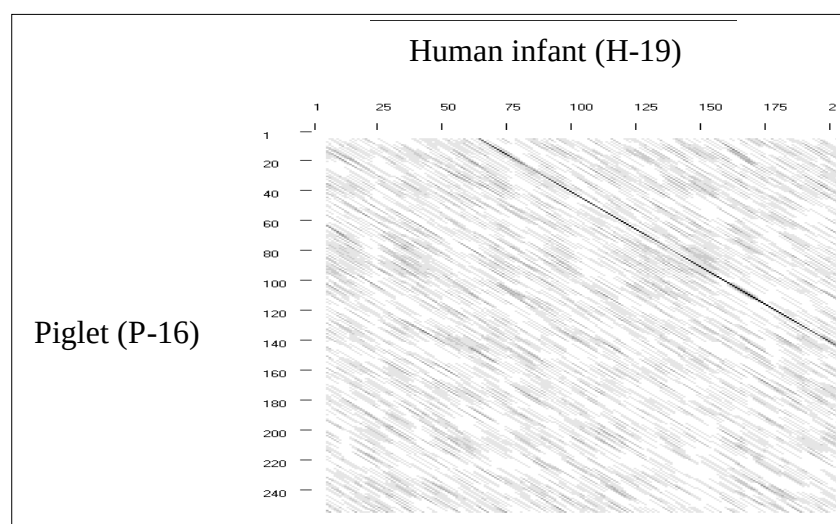


Fig 7: Dot plot matrix for translated amino acid of the VP7 gene.

revealed that certain porcine sequences showed greater similarity to human rotavirus strains than porcine RVA strains (Lv *et al.*, 2024).

CONCLUSION

Research on cross-species RVA sheds light on mutations that cause host adaptability and zoonotic transmission, which advances our knowledge of rotavirus molecular epidemiology and vaccine development. The findings of our study indicate that piglets and human infants are frequently infected with RVA and that the strains are highly varied, suggesting possible zoonotic transmission. Our research also suggested that, the rotavirus is developing through interspecies interaction, there may be a chance of a future outbreak. Thus, for a more thorough understanding

of the epidemiology of rotaviral infection, ongoing surveillance of group A rotavirus strains is necessary.

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Conflict of interest

The authors declare that they have no conflict of interest.

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