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Research Article

DETECTION AND GENOTYPIC CHARACTERIZATION OF ROTAVIRUS FROM DIARRHOEIC PIGLETS OF ARUNACHAL PRADESH, A NORTH EASTERN STATE OF INDIA

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Abstract- Rotavirus (RV) has been considered as one of the most important cause of severe gastroenteritis among human infants and the neonates of most farm animal species worldwide. In piglets, RV diarrhoea has been identified as a major cause of mortality and morbidity. The present study was undertaken to detect rotavirus from diarrhoeic faecal samples of pig population of Arunachal Pradesh and to investigate genotypic characterization of the same. A total of 342 faecal samples were collected from diarrhoeic piglets of different places of Arunachal Pradesh. The samples were screened for presence of RV by RNA-PAGE and RT-PCR with amplification of VP4 and VP7 genes of RV. Further, genotyping of the RV circulating among the pig population of Arunachal Pradesh was carried out to find out the G- and P-types of RV detected from diarrhoeic piglets. Of 342 diarrhoeic faecal samples, 53 (15.49%) and 127 (37.13%) samples were found positive for group A RV (GARV) in RNA-PAGE and RT-PCR respectively. On genotyping, it was observed that, major G-P combination circulating among the pig population Arunachal Pradesh was G10P[7] (33.3%) followed by G10P[8](8.33%) and G9P[8] (8.33 %). Most of the samples (50.00 %)were found to have mixed type of G-P combinations. To the authors' knowledge, this is the first report of genotypic characterization of RV from Arunachal Pradesh, India.

Keywords- *Piglets, Group A rotavirus, RNA-PAGE, RT-PCR, Nested-multiplex PCR, G-genotyping, P-genotyping*

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Introduction

Rotavirus infection is the leading cause of moderate to severe acute diarrhoea in the young animals Since its discovery in 1973, Rotavirus (RV) has been established as the most common cause of acute gastroenteritis among human infants and the neonates of most farm animal species throughout the world [1, 2, 3]. In piglets 25-30% mortality is seen during the pre-weaning period [2]. Amongst infectious agents associated with neonatal enteric disease, RV, Coronavirus, Cryptosporidium, and Escherichia coli collectively are responsible for 75-95% of infection in which RV alone accounts for about 27-36% [2]. Rotavirus belongs to the genus Rotavirus under the family Reoviridae [4, 5]. They are non-enveloped, icosahedral particles consisting of eleven segments of double stranded RNA (dsRNA) enclosed in a triple-layered protein capsid [6].The two outer capsid proteins namely, VP4 and VP7, both of which are independently responsible for neutralization of the virus [7].Group A rotavirus was classified according to its combination of two kinds of serotype, namely G (glycoprotein) serotype determined by VP7 antigenicity and P (protease-sensitive protein) serotype associated with VP4 antigenicity [2, 8]. Currently, 27 G-genotypes (from G1 to G27) and 35 P-types (from P[1] to P[35]) have been described [9]. Arunachal Pradesh (28.2180° N, 94.7278° E), a north eastern state of India, is a tribal state where there is no any taboo attached to the farming of Pigs. Almost all rural household has minimum of one to two or more pigs in their backyard [10]. Pig meat (pork) is very popular among all the tribes of the state. Despite having enormous potential of pig farming in Arunachal Pradesh, due to lack of proper technical knowledge and guidance most of the pig farmers suffers heavy loss due to various kinds of diseases, of which neonatal diarrhea caused by RV is one of the most important disease in piglets. Prevalence of RV infections in animals have been well documented from different parts of India [11, 12, 13]. However, no data on distribution of RV among pig population of Arunachal are available as no systematic study has been carried out so far. Studies conducted in Assam, a neighboring state of Arunachal Pradesh, have clearly indicated presence of RV among pig population of the state [14, 15].In order to protect and reduce the incidence of the disease, epidemiological studies in Arunachal Pradesh is of utmost importance besides developing technologies for the virus isolation, identification and above all molecular characterization of the virus for future vaccine strategy. This study was conducted to determine presence and genotypic distribution of RV infection in pig population of Arunachal Pradesh, with a view to have some baseline information to formulate control measures.

Materials and methods

Faecal samples

A total of 342 faecal samples from diarrheic piglets were collected aseptically from different places of Arunachal Pradesh, India. All collected faecal samples were stored at -20°C until further processing. Approximately 1 g of sample was vortexed with 4 volumes of 0.1M PBS (pH= 7.4) and centrifuged in a refrigerated centrifuge machine (Sigma, Germany) at 12000 x g for 20 minutes at 4°C. The supernatant was collected and stored at -20°C until further use.

Detection of rotavirus in faecal samples

Initial screening of the processed samples for rotavirus was done by RNA PAGE followed by RT PCR of VP4 and VP7 genes.

Ribonucleic acid polyacrylamide gel electrophoresis (RNA PAGE)

Viral RNA was extracted from the faecal suspensions by phenol chloroform mixture [16] and 0.1 M sodium acetate buffer (pH 5.0) containing 1% (w/v) sodium dodecyl sulphate (SDS). RNA concentration was assessed by nanodrop spectrophotometer (ND-1000; Thermo Scientific, USA). The extracted RNA is then subjected to native RNA PAGE. The electrophoretic run was carried out at 100-120 volts for 2-3 hours using vertical gel electrophoresis apparatus (Biorad, USA). The genomic migration pattern was detected by silver-staining of the gel[16].

PCR for amplification of VP4 and VP7 gene of RV

The viral RNA was extracted from the processed samples using RNA Sure® Virus Kit (Genetix, Asia Biotech Pvt. Ltd., New Delhi) following the manufacturer's instructions. The extracted RNA was used as templates for amplification of VP4 and VP7 genes of RV or stored at -20°C until further use.

Primers

For PCR amplification of VP4 and VP7 genes of RV, previously published primers were used [17]. For genotyping of RV, type specific primers reported for most common genotypes were selected based on available literatures [18]. The sequences of the primers, their positions in the genomic segment and the prototype viruses from which the sequence taken are presented in [Table-1].

RT-PCR for VP4 and VP7 gene amplification

A reverse transcription PCR (RT-PCR) was used for amplification of full length VP4 and VP7 genes of RV as per method of Gouvea *et al*., (1990) [17]. Briefly, for VP4 amplification, in RT-1 reaction, denaturation of template and primers (Con3 and Con2) was done at 95°C for 5 min. in a thermal cycler (VERITI 96 wells, Applied Biosystems) and immediately chilled on ice. In RT-2 reaction, denatured dsRNA was then added to the reaction mixture consisting of 10 µl of the 5X reaction buffer, 1 µlof 10 mM deoxynucleoside triphosphate (10mM dNTP) mixture, 2 µl of 25 mM $MgSO_4$, 1 µl of MuLV Reverse Transcriptase enzyme (5U/µl), 1 µl of Taq Polymerase (5U/ µl) and 3 µl of dimethyl sulfoxide in a final volume of 50 µl and then preincubated at 48°C for 45 min followed by 35 cycles of 94°C for 1 min., 52°C for 2 min., and 68°C for 3 min. and a final extension of 68°C for 10 min. and wind up by hold at 4°C. The VP7 gene was also amplified similarly using the primer pair Beg9(Forward) and End9(Reverse) and the annealing temperature was kept at 42°C.

G and P typing of RV by nested multiplex PCR

Representative RV positive samples (n=12) were subjected G-typing, carried out with the VP7 upstream primer and common G-type specific primers as described by Gouvea *et al*., (1994) [18]. Briefly, 5 µl of undiluted first RT-PCR product was added to a reaction mixture and subjected to nested multiplex PCR amplification with a mixture of type specific primers G1 to G10 and an upstream primer sBeg9, with the following PCR cycles: Preincubation at 94°C for 10 min. and 25 cycles of 1 min. at 94°C, 2 min. 54°C and 1 min. at 72°C, followed by a final incubation at 72°C for 10 min. and wind up by hold at 4°C. Similarly, P-typing of the same samples were carried out with the VP4 upstream primer and P-type specific primers as reported by Gouvea *et al*., (1994) [18]. Briefly, 5 µl of undiluted first RT-PCR product was added to a reaction mixture and subjected to nested multiplex PCR amplification with a mixture of type specific primers P[1], P[2], P[3], P[4], P[5], P[6], P[7], P[8], P[9], P[10] and P[11] and an upstream primer Con2, by following the PCR cycles as reported by earlier workers [18, 19] with slight modification of the annealing temperature, which was set at 50°C, briefly, preincubation at 94°C for 5 min. and 25 cycles of 1 min. at 94°C, 2 min. 50°C and 2 min. at 72°C, followed by a final incubation at 72°C for 5 min. and wind up by hold at 4°C.

Analysis of PCR products

PCR products were analyzed in 1.5 % agarose gel containing ethidium bromide (0.5 µg/ml).The electrophoresis was carried out for 60 mins at 80 V. The amplicons were visualized under UV light using a UV transilluminator (DNR System, USA) to detect the presence of specific amplicons.

Results and Discussion

Rotavirus has been a major cause of mortality and morbidity in young ones of most farm animal species as well as in human infants [1]. In the present study, RV was detected in faecal samples of piglets of Arunachal Pradesh, a north eastern state of India by RNA –PAGE and RT-PCR. Further, a nested multiplex PCR method was used to determine the G and P genotype of RV. Results showed that of 342 faecal specimens collected from diarrhoeic piglets, 53 (15.49%)samples were found to be positive for RV in standard PAGE assays. All positive RV nucleic acid segments were separated in a clustered arrangement of 4:2:3:2, with segments 2, 3 and 4 migrating close together, segments 7, 8 and 9 closely spaced, and segments 10 and 11 as found in long RNA migration patterns indicating typical of group A mammalian RV [Fig-1]. In this study electropherotyping (RNA-PAGE) was selected as the primary method for detection of RV strains in diarrhoeic piglets, because each RV strain reveals a single distinct electropherotype upon PAGE and 11 segments of dsRNA by PAGE yield a pattern which is both constant and characteristic for a particular RV isolate [20]. Similar observations were also reported by earlier workers [21, 22, 23]. Detection of RV by RT-PCR showed a higher percentage of detection than RNA-PAGE. Out of 342 faecal samples collected, 127[37.13%] samples from diarrhoeic piglets were found to be positive for RV in standard RT-PCR. In RT-PCR, full length amplification of both VP4 and VP7 gene of RV could be obtained with a desired band sizes of 876 bp and 1062 bp respectively [Fig-2 and 3] in all positive samples.

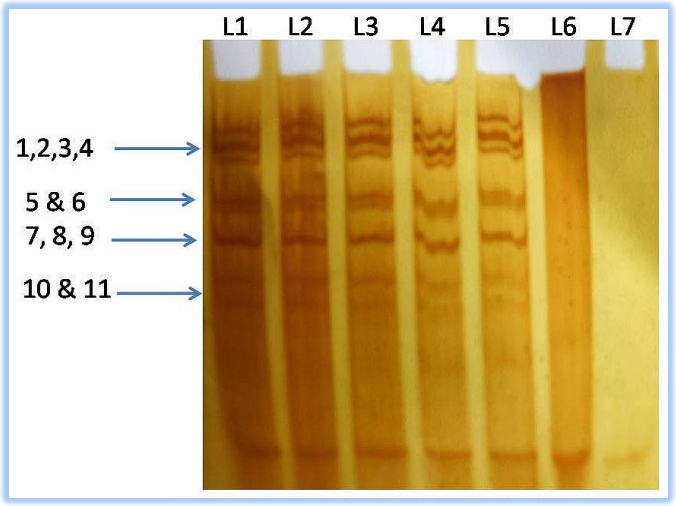

Fig-1 RNA Electrophoresis of Rotavirus RNA
L1:Positive control, L2-L5:Positive faecal samples, L6: Negative Control

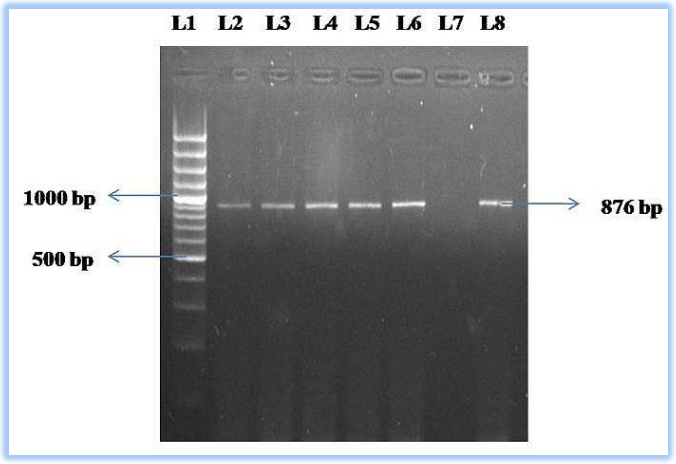

Fig-2 Amplification of VP4 region of porcine rotavirus
L1:100bp plus ladder, L2-L6:Positive faecal samples L7:NTC L8: Positive control (Porcine RV)

Table-1 Primers used in PCR for amplification of specific fragments from different genes of RV

Primer	Type of PCR	Sequence (5' -3')	Position	Reference
VP7 gene amplification				
Beg9 (+)	RT-PCR	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	[17]
End9 (-)		GGTCACATCATACAATTCTAATCTAATCTAAG	1062-1036	
G-typing				
9con1	Nested-multiplex PCR	TAG CTC CTT TTA ATG TAT GG	37-56	[18]
9T-1-G1		TCT TGT CAA AGC AAA TAA TG	176–195	
9T-2-G2		GTT AGA AAT GAT TCT CCA CT	262–281	
9T-3-G3		GTC CAG TTG CAG TGT AGC	484–501	
9T-4-G4		GGG TCG ATG GAA AAT TCT	423–440	
9T-9-G9		TAT AAA GTC CAT TGC AC	131–147	
VP4 gene amplification				
Con3 (+)	RT-PCR	TGGCTTCGCTCATTTATAGACA	11-32	[19]
Con2 (-)		ATTTCGGACCATTTATAACC	868-887	
P-typing				
pUK-P[5]	Nested-multiplex PCR	GCCAGGTGTCGCATCAGAG	336-354	[17]
PGott-P[6]		GCTTCAACGTCCTTTAACATCAG	465-487	
1T-1-P[8]		TCTACTTGGATAACGTGC	339-356	[19]
2T-1-P[4]		CTATTGTTAGAGGTTAGAGTC	474-494	
3T-1-P[6]		TGTTGATTAGTTGGATTCAA	259-278	
4T-1-P[9]		TGAGACATGCAATTGGAC	385-402	
5T-1-P[10]		ATCATAGTTAGTAGTCGG	575-594	
pB223-P[11]		GGAACGTATTCTAATCCGGTG	574-594	[35]

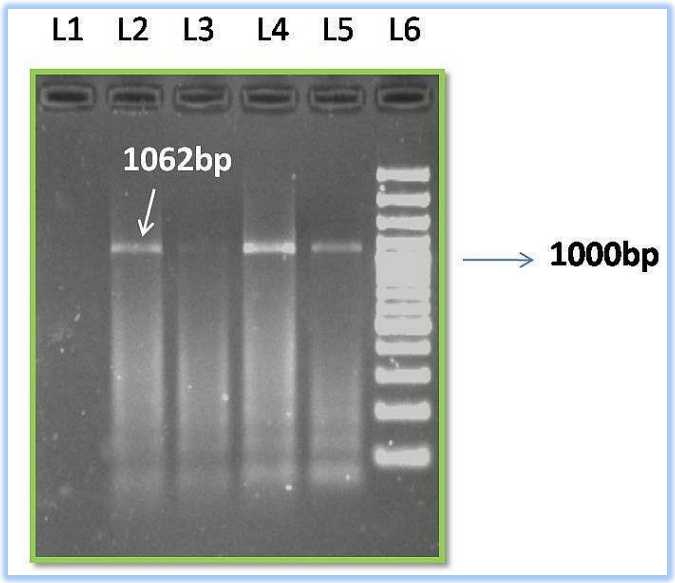

Fig-3 Amplification of VP7 region of porcine rotavirus

L1:NTC, L2-L4:Positive faecal samples, L5:Positive control (Porcine RV) L6: 100bp plus ladder

Efficacy of RT-PCR over RNA-PAGE in detection of RV in diarrhoeic faecal samples have been reported earlier [3, 24]. Although RNA-PAGE has been considered as 'gold standard' for detection of RV [25], it was observed that RT-PCR in the present study to be more effective and showed higher sensitivity than RNA-PAGE. This might be attributed to the nucleic acid-based techniques such as RT-PCR which could detect a very minute concentration of the virus that may escape detection by RNA-PAGE. Based on antigenicity of outer capsid proteins of RV, VP7 and VP4, two independent genotype *viz*. G-genotype and P-genotype have been established. Accurate detection and genotyping of RV is important for several reasons. The epidemiology of RV infection shows significant geographic differences, so global surveillance is essential to detect the prevalence and incidence of different RV genotypes. In the present study also, representative (n=12) samples were subjected to G- and P- typing by nested multiplex PCR. In G- genotyping, it was observed that, of 12 samples subjected for genotyping, G10 (5/12, 41.66%) genotype was predominant followed by G1 (2/12, 16.66 %) and G9 (1/12, 8.33%) with the expected PCR products of 715bp (G10), 159bp (G1) and 111bp (G9) respectively (data not shown). Some (4/12, 33.33%) of the samples were found to have mixed genotype or non-typable genotype as evident by multiple amplification band in the typing PCR. (data not shown). In P- genotyping, it was observed that, of 12 samples subjected for genotyping, P[7] (7/12, 58.33%) genotype was predominant followed by P[8] (2/12, 16.66 %) a with the expected PCR products of 502bp (P[7]),) and 345bp (P[8]) respectively (data not shown). Some (4/12, 33.33%) of the samples were found to have mixed genotype or non-typable genotype as evident by multiple amplification band in the typing PCR. (data not shown).

In the present study, genotypic characterization revealed distribution of G10P[7], G10P[8], and G9P[8] of G-P genotype combinations in piglets of Arunachal Pradesh. Global epidemiologic surveys have identified G3, G4, G5 and G11 as the most common G genotypes and P[6] and P[7] as the most common P genotypes associated with diarrhoea in pigs. Also, rare genotypes (G1, G2-like, G6, G8, G9, G10, and G12) were reported in pigs which are commonly associated with human and cattle [3, 7, 18, 26, 27, 28, 29, 30]. Similarly, apart from the common porcine P genotypes (P[6] and P[7]), rare and/or novel P genotypes (P[1], P[5], P[8], P[11], P[13], P[14], P[19], P[23], P[26]) were identified in pigs [3, 29].In India, 4 types of G genotypes (G4, G6, G9, G12) and 4 types of P genotypes (P [6], P [7], P [13], P[19]) of RV As have been detected from pigs so far [14]. In India, the prevalence of RV infection in pigs can be as high as 25.7% [31]. In addition, unusual RV strains of porcine origin (G1P[19], G9P[6], G9P[19] G4P[4] and G4P[6]) have been reported in India in several studies [30, 31, 32, 33, 34]. The present findings are also in agreement with the findings of earlier workers.

Conclusion

As the molecular epidemiology of rotavirus developed recently, genetic typing based on VP7 gene andVP4 gene, G-genotype and P-genotype, respectively, are being used frequently to understand the epidemiology of RV in a particular geographical area. Earlier to this, no report on RV genotyping has been reported from Arunachal Pradesh. Findings of the present study will help in formulating the control strategies against RV induced diarrhea in pig population of Arunachal Pradesh so as to reduce the economic loss and increase the profitability of the enterprise.

Application of research

Rotavirus (RV) has emerged as one of the important causes of piglet diarrhoea. Arunachal Pradesh being a tribal state, almost all rural household has minimum of one to two or more pigs in their backyard. It is one of the most economical and beneficial enterprise among the poor farmers of the state. So, findings of the present study will help in controlling the RV infection in pig population of the state by formulating appropriate strategies. Further studies are necessary to carry out genotyping characterization of circulating RVs with more number of samples and to find out the novel strains of RV, if any.

Research Category: Animal Viruses, Neonatal Diarrhoea

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Author Contributions: This study was a part of GBG's research work during his M.V.Sc. program. GBG carried out the experiment. DPB, SKD and YPSM designed the experiment and provided necessary guidelines. MB and SPA assisted in laboratory works. DPB drafted the final manuscript.

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Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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