Circulation of group A rotaviruses among neonates of human, cow and pig: study from Assam, a north eastern state of India

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Abstract Rotavirus (RV) infections are worldwide in distribution causing high morbidity and mortality in human and animal neonates. Human settlements in close proximity of animals aids for genetic re-assortment of the virus by interspecies transmission and consequent emergence of new viral antigenic strain. Therefore, the present study was designed to explore RV incidence in a single approach from human and animal neonates sharing similar environment. Altogether, 200 diarrheal samples from children (50), piglets (80) and calves (70) were collected during the year of 2010–2012 from various locality, farms and hospitals, initially screened through monoclonal antibody based enzyme immunoassay followed by RNA-PAGE and VP7 gene amplification by Reverse transcription PCR. The overall prevalence of rotavirus was found to be 41.5 % (83/200) where maximum numbers of positive cases were found in piglets (46.3 %) followed by human (40 %) and cow (37.1 %). Majority of samples demonstrated characteristic group A rotavirus (RVA) electropherotype of 4:2:3:2 pattern. Moreover, RNA profiles of seven samples from piglets and calves revealed variation in the migration pattern of class II, III and class IV segments. The study, for the first time from the valley, detected 43.7 % of neonatal RVA positive cases from human and animal sharing similar setting. The variation in RNA migration pattern in seven cases signifies tentative cases of gene re-assortment that warrant further evaluation.

Keywords Electropherotype · Neonates · Reassortment · Rotavirus · VP7

Introduction

Rotavirus (RV) infections are the leading cause of neonatal diarrhea and are worldwide in distribution causing high morbidity and mortality in humans, animals and birds [1, 4, 14, 21]. Among all serogroups, rotaviruses belonging to group A are predominant cause of severe gastroenteritis [4, 26]. This non enveloped virus of family Reoviridae, having a double-stranded RNA (dsRNA) genome of 11 segments can exchange (reassort) genes [6, 7, 12] and the mechanism is very often in a setting where close proximity among human and animal exhibit, thus, aids for genetic reassortment [3]. The socio-economic status of India is typically based on agriculture and rearing farm animals. These animals act as an important host to thrive the infection ensuing possibility of interspecies transmission by fecal-oral route that accounts the basis of rotaviral circulation in the whole community. This phenomenon is a feature that is predicted to generate possibly more dangerous virus strains and successively may cause serious threat to the existing vaccine as the efficacy and effectiveness of RV vaccine may differ depending on the predominant natural strain types [17].

Many studies [9, 13, 22, 23] illustrated on detection and typing of RV by various serological and molecular techniques that has improved our perceptive of the diversity of RV strains where antibody based technique like enzyme immunoassay (EIA) and the molecular techniques like polyacrylamide gel electrophoresis (PAGE), reverse transcriptase polymerase chain reaction (RT-PCR) etc. are widely used. Presently, there are seven RV groups (A–G) and at least four subgroups (SGI, SGII, SGI+II, and SGnon-I/non-II)

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within group A that have been recognized [2]. The RNA segments separated on polyacrylamide gel generates that can be broadly divided into “long,” “short,” and “abnormal” RNA profiles, based on the relative migration of RNA segments [8]. Genotype-specific detection or classification is based on reverse transcription followed by polymerase chain reaction (RT-PCR) and nucleotide sequence analysis of VP7 and VP4 genes. The VP7 (glycoprotein) and VP4 (proteases) proteins form the smooth outer capsid (G serotype) and short spike (P genotype), respectively, and are the major antigens inducing neutralizing immune responses during RV infections. Based on molecular differences, currently, 35 P-types and 27 G-types have been reported, of these, 15 P-types and 12 G-types have been identified in human infections [12, 19, 20, 25].

More recent research [20, 21, 28] has occurred in the field where genetic variability study provided functional data for a better understanding of virus epidemiology, particularly with respect to evidence of interspecies transmission of rotaviruses which increases the possibility of emerging new strains and unusual genotypic combinations. This type of research also focuses the development and monitoring of effective vaccines. Maintenance of domestic animals for living and trade is very common in north-east India, and animals and humans usually live in close proximity and share (in some cases) same water bodies in rural settings. This condition provides a perfect opportunity for interspecies transmission and genetic re-assortment. Moreover, there are no results in the literature regarding combined study for detecting RV circulating among humans and animals sharing single locality, especially from this part of India. The present study was, therefore, intended to ascertain the occurrence of rotaviruses in human and animal neonates associated closely and to characterize the field samples testing positive for RV by EIA, RNA-PAGE and RT-PCR methods. This study will highlight the prevalence of RV incidence cases and occurrence of variant strains circulating among human and animals at a single location that would have implications in diarrhea surveillance and vaccine evaluation program.

Materials and methods

Sample collection

The area for sampling was characteristically chosen having a close association of animals and human. The Nepali basti of Guwahati (26.1838°N, 91.7633°E), India displayed the exact state of affairs where unorganized cattle farms are maintained by the dwellers. Likewise, in a sole setting, the intake of house hold wastes by the pigs also assists interspecies transmission where the associated neonates, at both

cases are at risk, thus satisfying the principle of study. A total of 200 diarrheal stool samples from piglets (80), calves (70) and children (50) were collected from freshly voided faeces or obtained by rectal evacuation during the year 2010–2012. In addition, human samples from clinically affected children under the age group of <5 years having history of involvement with farm animals were collected from the diarrheal ward of Department of Pediatrics and Neonatology, Guwahati Medical College Hospital (GMCH), Assam, India. The pig and cattle farms of College of Veterinary Science, Khanapara and NRC on Pigs, Rani, Guwahati, Assam, India respectively were also explored, being organized farms for collecting diarrheal fecal samples where infectivity is restored within the environment causing threat to the tenants. Collected fecal samples were transported in a ice-chamber within 2 h of sampling to the testing laboratory (Department of Microbiology, College of Veterinary Science, AAU, Khanapara) and 20 % suspension of each sample was made in 0.06 M PBS (NaCl, KCl, Na₂HPO₄, KH₂PO₄), pH 7.2. After clarification at 12,000 g for 20 min in refrigerated centrifuge machine (Biofuge, Germany), the supernatant was collected and preserved at −20 °C till further use.

Enzyme immunoassay

Suspected field samples were individually screened for RV antigen using a monoclonal antibody (mAb) directed against product of sixth viral gene (VP6) using sandwich type enzyme immunoassay (mAb-EIA) kit (Premier Rotacclone; Meridian Bioscience Inc., Cincinnati, OH) according to the manufacturer's guidelines.

Polyacrylamide gel electrophoresis

20 % fecal suspension was used for viral RNA genome extraction using phenol chloroform mixture [15] and 0.1 M sodium acetate buffer (pH 5.0) containing 1 % (w/v) sodium dodecyl sulphate (SDS). RNA quantity and quality was assessed using a Nanodrop Spectrophotometer (ND-1000; Thermo Scientific, USA) and was analyzed by RNA-PAGE in 7.5 % polyacrylamide slab gels using 3 % spacer gel in a discontinuous buffer system [18] without SDS. Electrophoresis was carried out at 70–100 V for 2–3 h in room temperature using vertical slab gel electrophoresis apparatus (Biorad, USA). The gel was further stained with silver nitrate (AgNO₃) solution to generate electrophoretic type of the fragmented viral RNA [27].

VP7 gene amplification by RT-PCR

Reverse transcription of suspected fecal samples for amplification of full length VP7 gene was performed for

which viral RNA was extracted using commercial kit (Axygen, USA) following manufacturer's protocol and was used as templates in RT-PCR. One step RT-PCR was performed using set of generic primers as described earlier [13] in two reaction steps holding a total reaction volume of 50 μ l. In RT-1 reaction, the mixture of template and 10 pmol primer was denatured at 95 °C for 5 min in a thermal cycler (VERITI 96 wells, Applied Biosystems) and immediately chilled on ice. RT-2 reaction for cDNA synthesis and gene amplification was performed in the same tube by adding 5 $\times$ RT buffer, 10 mM dNTP mix, 25 mM MgSO₄, 5U of AMV RT enzyme, 5U of Taq polymerase (Invitrogen) and DMSO under following thermal cycling profile: Preincubation at 48 °C for 45 min, denaturation at 94 °C for 1 min, annealing at 46 °C for 2 min, extension at 68 °C for 3 min and repeated the above steps for 39 cycles following final extension for 10 min at 68 °C and wind up by hold at 4 °C. The PCR amplified product was confirmed by agarose gel electrophoresis using 1.5 % agarose (Amresco) containing ethidium bromide (0.5 mg/ml) in 0.5 $\times$ tris borate EDTA (TBE) buffer and the gel was visualized under UV transilluminator (Kodak, Biostep, Germany).

Results

Cases of diarrhea outbreak in both locality and hospitals were regularly attended to comprehend clinical symptoms followed by sample collection. The sampling area along with individual patients from hospital showed a history of unhygienic and low economic scenario. The affected animals showed diarrhea and occasional vomiting with severe and mild dehydration. Out of 200 suspected field samples from piglets, calves and children, the overall prevalence of RV was found to be 41.5 % (83/200) where maximum numbers of positive cases were found in piglets (46.3 %) followed by human (40 %) and cow (37.1 %). The children from the area where there is close association with animals demonstrated 46.4 % (13/28) of RV positive cases and

31.8 % (7/22) positivity were recorded for hospitalized patients. The details of RV infection in three different hosts during the course of study were presented in Table 1.

Figure 1 Each RV strain reveals a single distinct pattern of electropherotype upon RNA-PAGE. Analysis of the electrophoretic mobility of the 11 segments of dsRNA yields a pattern which is both constant and characteristic for a particular RV isolate. The electropherotypes of the RV strains from diarrheic piglets, calves and children generated after RNA-PAGE were compared with standard reference strain (Fig. 2, Lane 1) and corresponded to the characteristic migration pattern of 4:2:3:2, which confirms the presence of group A rotavirus (RVA). All the suspected field samples were compared with standard reference strain where five samples of piglets of age 2–4 months and two samples of cow calves of age 3–4 months collected from an organized farm demonstrated additional variation in the migration pattern of dsRNA. Co-electrophoresis of reference strain (Fig. 2, Lane 1) and four strains of piglets (two samples shown here in Fig. 2, Lane 2 & 3,) from organized farm revealed longer electropherotype and fast migrating class III segments (7–9) and class IV segments (10–11). However, one strain (Fig. 2, Lane 6) from piglet has demonstrated shorter electropherotype. Moreover, class II segment of two cow calves (one shown here in Fig. 2, Lane 5) from unorganized farm was found to be slow migrating as compared to the other strains. All the field samples, except few (due to low sample amount), were processed for amplification of VP7 region that yielded an expected band of 1062 bp for pig and human RV's (Fig. 3) and 1011 bp for bovine RV strains (Fig. 4).

Discussion

The primary aim of the study was to detect RV from neonates of human, cow and pig characteristically from a setting which is closely shared. Those setting facilitate interspecies transmission that acts as key aspect for genetic

Table 1 Rotavirus infection in three different hosts

Species	Year of study	Community/location	Symptoms	Screening ^d	No. tested (<i>n</i>)	Positive		Prevalent sub-group ^c
						<i>n</i> ^a	% ^b	
Pig	2010–11	Rural	Symptomatic	ELISA, PAGE, PCR	80	37	46.3	SGII
Cow	2010–11	Rural	Asymptomatic	ELISA, PAGE, PCR	70	26	37.1	SGI
Human	2011–12	Urban/rural	Symptomatic	ELISA, PAGE, PCR	Outpatient	28	13	46.4
					Nasocomial	22	7	31.8

^a No. of samples

^b Prevalence in percentage

^c Sub-grouping based on RNA migration

^d Enzyme linked immunosorbant assay, polyacrylamide gel electrophoresis of RNA segments, reverse transcriptase polymerase chain reaction

reassortment producing viable new strains. In our study, sampling area demonstrated close proximity among humans and animals where barely hygienic conditions are upheld. The monoclonal antibody (mAb-EIA) and nucleic acid (RT-PCR) based detection allowed significant and rapid diagnosis of rotaviral infection from the clinical samples. The infection was found more prevalent during the first week of life and the positivity rate was highest with whitish watery/liquid faeces. Out of 200 suspected field samples from piglets, calves and children, the overall prevalence of RV was found to be higher in piglets followed by humans and calves. Overall, RVAs were detected more recurrently and is consistent with the earlier reports

Fig. 1 Showing comparative evaluation (in percentage) of mAb-EIA, RNA-PAGE and RT-PCR performed for rotavirus detection from field samples. RT-PCR is showing highest positivity followed by mAb-EIA and RNA-PAGE

Fig. 2 RNA-PAGE analyses of rotavirus 11 segmented dsRNA that exhibited typical cluster of 4:2:3:2 pattern for group A strains from stool suspensions of human, cattle and pig neonates with acute diarrhea are shown. Numbers denote positions and class of rotavirus dsRNA segments. *Lane1*, Standard pig RV; *lane2*, Rv/P-2 and *lane3*, Rv/P-6 showing fast migrating class III and class IV segments. *Lane4*, pool of stool suspensions taken from healthy animal. *Lane5*, Rv/Bo-112 showing shorter electropherotype; *lane6*, Rv/P-69 showing shorter electropherotype

[10, 12, 19, 21]. Past studies [23] from the valley, solely on pigs also confirmed piglet RV prevalence. In cows and humans, however, positive cases of 38.2 and 44 % respectively are the first reported incidence from this region. Human neonatal samples collected from the combined locality as well as from children of the dwellers associated with organized farms represented maximum rate of RV infection than the hospital patients. This clearly connotes that RV transmission from animal origin to human population is maximum in close setting.

The discontinuous RNA-PAGE was employed for RV strain grouping and studies also reported that this technique is another mostly used gold standard test for RV detection in faeces [2, 5, 11, 16, 24]. Analysis of electrophoretic mobility of the 11 segments of RV dsRNA by PAGE corresponded to the basic pattern of 4:2:3:2 typical for group A rotavirus (RVA). The result of the present study also represented some additional variation in the migration pattern of the RNA segments of seven viral isolates where viral strains from four piglets of organized farms exhibited longer electropherotype mold, however from the same area/farm, one piglet RV strain

Fig. 3 Amplification of the VP7 genes by RT-PCR. Amplification products corresponding to the VP7 from pig (*lanes 2, 3 and 4*) and human (*5, 6 and 8*) samples are shown. *LaneM*, molecular weight markers (100-bp ladder); *lane7*, negative control. The size of amplified bands is indicated

Fig. 4 Amplification of the VP7 genes by RT-PCR. Amplification products corresponding to the VP7 from bovine samples (*lanes 3, 4, 5, 6 and 7*) of six different diarrhea infected are shown. *Lane1*, molecular weight markers (100-bp ladder); *lane2*, negative control. The size of amplified bands is indicated

has demonstrated shorter electropherotype. Likewise, detection of two rotaviral strain from cow calves of similar region revealed slow migrating class II segments. Nevertheless, variations in the RNA profiles of human samples were not detected or apparent during the study but the entire phenomenon might be responsible for the circulation of these variant strains within the human community too. Thus, circulations of variant strains of RV inside a single region are most likely suggesting presence of genetic diversity of RV. The VP7 gene or ninth segment of RV genome encodes a glycoprotein that forms the outer surface of the virion and induces neutralizing immune responses during RV infections. This gene, targeted by RT-PCR amplified an expected band of 1062 bp for pig and human RV's; 1011 bp for cow RV strains and further confirmed the presence of RV in the studied species as been evidenced earlier too [2, 25].

Circulations of RV in different susceptible host in a single geographical region therefore generate a potential possibility of zoonosis and thus provokes for reassortment of the viral strains consequently causing severe menace to vaccine failure. The present study detected 83 positive cases (41.5 %) of neonatal RVA incidence amongst man and animals from related vicinity for the first time from this region. The significant variation found in the RNA pattern of the RV strains in few samples implies circulation of various strains and chances of gene reassortment. The present study, however, could not surmise about the genotypes of the circulating strains detected during the course of study owing deficient in sequence data. Therefore, the RVA positive samples will be further used for G-P typing and phylogenetic studies to know their genetic make-up and prevalence of RV strain in different hosts at a single location. This will help out in continuous surveillance of RV incidence as well as to provide information about the occurrence of novel strains.

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References

- Ahmed S, Hussain M, Akhter S, Islam T, Ahmed SU, Kabir ML. Genotypes of rotavirus diarrhoea in a children hospital of Bangladesh. *Mymensingh Med J.* 2012;21:497–502.
- Aminu M, Page NA, Ahmad AA, Umoh JU, Dewar J, Steele AD. Diversity of rotavirus VP7 and VP4 genotypes in Northwestern Nigeria. *J Infect Dis.* 2010;202(Suppl):S198–204.
- Armah GE, Hoshino Y, Santos N, Binka F, Damanka S, Adjei R, Honma S, Tatsumi M, Manful T, Anto F. The global spread of rotavirus G10 strains: Detection in Ghanaian children hospitalized with diarrhea. *J Infect Dis.* 2010;202(Suppl):S231–8.
- Badaracco A, Garaicoechea L, Rodriguez D, Uriarte EL, Odeon A, Bilbao G, Galarza R, Abdala A, Fernandez F, Parreno V. Bovine rotavirus strains circulating in beef and dairy herds in Argentina from 2004 to 2010. *Vet Microbiol.* 2012;158:394–9.
- Bozdayi G, Dogan B, Dalgic B, Bostanci I, Sari S, Battaloglu NO, Rota S, Dallar Y, Nishizono A, Nakagomi O, Ahmed K. Diversity of human rotavirus G9 among children in Turkey. *J Med Virol.* 2008;80:733–40.
- Broor S, Ghosh D, Mathur P. Molecular epidemiology of rotaviruses in India. *Indian J Med res.* 2003;118:59–67.
- Chouikha A, Fredj MB, Fodha I, Mathlouthi I, Ardhaoui M, Teleb N, Brini I, Messaadi F, Mastouri M, Sfar T, Hachicha M, Kammoun T, Bouaaziz A, Amri F, Harbi A, Zribi M, Bousnina S, Khemakhem A, Boujaafar N, Trabelsi A, Steele AD. Evolution of group A rotavirus strains circulating in Tunisia over a 3-year period (2005–2007). *Pathol-biol.* 2011;59:e79–83.
- Desselberger U, Iturriza-Gomara M, Gray JJ. Rotavirus epidemiology and surveillance. *Novartis Found Symp.* 2001;238:125–47 discussion 147–152.
- Falcone E, Tarantino M, Di Trani L, Cordioli P, Lavazza A, Tollis M. Determination of bovine rotavirus G and P serotypes in Italy by PCR. *J Clin Microbiol.* 1999;37:3879–82.
- Ganime AC, Carvalho Costa FA, Mendonca MC, Vieira CB, Santos M, Costa Filho R, Miagostovich MP, Leite JP. Group A rotavirus detection on environmental surfaces in a hospital intensive care unit. *Am J Infect Control.* 2012;40:544–7.
- Gatti MS, Ferraz MM, Racz ML, de Castro AF. Rotavirus excretion in naturally infected pigs with and without diarrhoea. *Vet Microbiol.* 1993;37:187–90.
- Ghosh S, Samajdar S, Sinha M, Kobayashi N, Taniguchi K, Naik TN. Molecular characterization of rare bovine group A rotavirus G15P[11] and G15P[21] strains from eastern India: identification of simian SA11-like VP6 genes in G15P[21] strains. *Virus Genes.* 2008;37:241–9.
- Gouvea V, Glass RI, Woods P, Taniguchi K, Clark HF, Forrester B, Fang ZY. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J Clin Microbiol.* 1990;28:276–82.
- Gunn L, Feeney SA, Cashman O, Collins PJ, Coyle PV, O'Shea H. Molecular characterization of group A rotavirus found in elderly patients in Ireland; Predominance of G1P[8], continued presence of G9P[8], and emergence of G2P[4]. *J Med Virol.* 2012;84:2008–17.
- Herring AJ, Inglis NF, Ojeh CK, Snodgrass DR, Menzies JD. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *J Clin Microbiol.* 1982;16:473–7.
- Jindal SR, Maiti NK, Oberoi MS. Genomic diversity and prevalence of Rotavirus in cow and buffalo calves in northern India. *Rev Sci Tech.* 2000;19:871–6.
- Kirkwood CD. Genetic and antigenic diversity of human rotaviruses: potential impact on vaccination programs. *J Infect Dis.* 2010;202(Suppl):S43–8.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680–5.
- Martella V, Ciarlet M, Banyai K, Lorusso E, Arista S, Lavazza A, Pezzotti G, Decaro N, Cavalli A, Lucente MS, Corrente M, Elia G, Camero M, Tempesta M, Buonavoglia C. Identification of group A porcine rotavirus strains bearing a novel VP4 (P) Genotype in Italian swine herds. *J Clin Microbiol.* 2007;45:577–80.
- Matthijns J, De Grazia S, Piessens J, Heylen E, Zeller M, Giammanco GM, Banyai K, Buonavoglia C, Ciarlet M, Martella V, Van Ranst M. Multiple reassortment and interspecies transmission events contribute to the diversity of feline, canine and feline/canine-like human group A rotavirus strains. *Infect Genet Evol.* 2011;11:1396–406.

21. Miyazaki A, Kuga K, Suzuki T, Kohmoto M, Katsuda K, Tsunemitsu H. Genetic diversity of group A rotaviruses associated with repeated outbreaks of diarrhea in a farrow-to-finish farm: identification of a porcine rotavirus strain bearing a novel VP7 genotype, G26. *Vet Res.* 2011;42:112.
22. Mukherjee A, Nayak MK, Roy T, Ghosh S, Naik TN, Kobayashi N, Chawla Sarkar M. Detection of human G10 rotavirus strains with similarity to bovine and bovine-like equine strains from untypable samples. *Infect Genet Evol.* 2012;12:467–70.
23. Neog BK, Barman NN, Bora DP, Dey SC, Chakraborty A. Experimental infection of pigs with group A rotavirus and enterotoxigenic *Escherichia coli* in India: gross, histopathological and immunopathological study. *Vet Ital.* 2011;47:117–28.
24. Ramos AP, Stefanelli CC, Linhares RE, de Brito BG, Santos N, Gouvea V, de Cassia Lima R, Nozawa C. The stability of porcine rotavirus in feces. *Vet Microbiol.* 2000;71:1–8.
25. Rao CD, Gowda K, Reddy BS. Sequence analysis of VP4 and VP7 genes of nontypeable strains identifies a new pair of outer capsid proteins representing novel P and G genotypes in bovine rotaviruses. *Virology.* 2000;276:104–13.
26. Soeorg H, Tamm E, Huik K, Pauskar M, Magi D, Pruudel K, Vainomae L, Moosar L, Kirss K, Torm S, Narska M, Putsepp A, Nurm H, Pruunsild K, Janes A, Zilmer K, Lutsar I. Group A rotavirus genotypes circulating prior to implementation of a National Immunization Program in Estonia. *Hum vaccin immunother.* 2012;8:465–9.
27. Svensson L, Uhnöo I, Grandien M, Wadell G. Molecular epidemiology of rotavirus infections in Uppsala, Sweden, 1981: disappearance of a predominant electropherotype. *J Med Virol.* 1986;18:101–11.
28. Tatte VS, Chitambar SD. Diversity in the VP7 encoding genes of rotavirus strains isolated from adolescent and adult cases of acute gastroenteritis. *J Med Virol.* 2012;84:1481–8.