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Molecular analysis of non structural rotavirus group A enterotoxin gene of bovine origin from India

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ABSTRACT

The rotavirus enterotoxin NSP4 (nonstructural protein 4), plays a pivotal role in viral morphogenesis as well as pathogenesis. In this study, the NSP4 gene of rotavirus group A (RVA) isolates of bovine origin isolated in several states of India from 2008 to 2011 were characterized. The complete open reading frame of 23 RVA strains were sequenced and analyzed phylogenetically. Genotype E1 was detected for the first time in bovines from India, in addition to the more common bovine genotype E2. Sequence similarity analysis of the E1 sequences showed a close genetic relatedness to human strains. Six of the bovine E2 genotypes strains clustered near bovine and unusual human strains (possible human animal reassortant) from Thailand, while the remaining E2 sequences clustered with Indian bovine strains. Analysis pointed out one positively selected site (154aa), believe to be part of an antigenic region and 123 negatively selected sites. Unexpectedly, a pentameric NSP4 structure of the coiled coil domain in the E1 carrying strains and a monomeric NSP4 in RVA strain P14 (E2) was predicted based on homology modeling, potentially affecting the biological properties of NSP4. The close relationship between bovine and human rotavirus strains further highlights the complex interaction among rotaviruses of different species.

1. Introduction

Group A rotaviruses (RVA) are a very common cause of diarrheal diseases worldwide, causing a high morbidity and mortality among humans and huge economic losses in animal industry (Estes and Kapikian, 2007). Rotaviruses (RVs) belong to the family *Reoviridae*, and possess a genome of 11 segments of double stranded RNA, encoding six structural and six nonstructural proteins. The identification of the nonstructural protein 4 (NSP4) as the first viral enterotoxin (Ball et al., 1996) has increased scientific interest to better understand its structure, sequence variability among RVAs infecting different species and mechanism of action in inducing secretory diarrhea in suckling mice.

NSP4 is a 175 amino acids transmembrane, endoplasmic reticulum (ER) specific glycoprotein with pleotropic functions and contains an uncleaved signal sequence at the N terminus followed by three hydrophobic domains namely H1 (7–21 aa), H2 (29–47 aa) and H3 (67–85 aa) and a coiled α helical domain (95–137 aa) (Estes, 2001). The amino terminal region (1–44 aa) of the gene is located in the lumen of the endoplasmic reticulum, whereas its carboxyl terminal region (45–175 aa) constitutes the cytoplasmic tail (CT) that exhibits all the known important biological properties associated with the protein which include alteration of Ca^{2+} homeostasis by the release of Ca^{2+} from the endoplasmic reticulum (Dong et al., 1997; Hyser et al., 2010; Tian et al., 1994), membrane permeabilization (Newton et al., 1997), Ca^{2+} and VP4 binding

Abbreviations: RVA, rotavirus group A; NSP4, nonstructural protein 4; RVs, rotaviruses; aa, amino acid; nt, nucleotide; CT, cytoplasmic tail; NCBI, The National Center for Biotechnology Information; NJ, Neighbor Joining; SLAC, Single Likelihood Ancestor Counting; FEL, Fixed Effect Likelihood; MEME, Mixed Effects Model of Evolution; FUBAR, Fast Unbiased Bayesian AppRoximation; NCDV, nebraska calf diarrhea virus.

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(Estes and Kapikian, 2007), double layered particle interaction for transport in the lumen of the endoplasmic reticulum for maturation into triple layered particles (Au et al., 1993; O'Brien et al., 2000) and diarrhea induction in newborn mouse pups (Ball et al., 1996; Horie et al., 1999; Jagannath et al., 2006).

At least 15 E genotypes (E1–E15) based on an 85% identity cut off values for the NSP4 gene have been identified so far (Matthijnssens et al., 2011; Papp et al., 2012). The RVA genotypes E1 (Wa like), E2 (DS-1 like) and E3 (AU-1), previously known as genotypes B, A and C, respectively have been detected in humans and animals (Matthijnssens et al., 2011). Each NSP4 genotype appears to segregate more or less according to the RVA host species (Ciarlet et al., 2000) suggestive of predominance of a particular genotype in a particular species. The rotavirus NSP4 sequences have been analyzed in more detail for human (Araújo et al., 2007; Ben et al., 2012; González Ochoa et al., 2013; Tavares et al., 2008) and avian species (Mori et al., 2002), but bovine species remains limitedly studied until now. In this study, 23 NSP4 sequences of bovine RVA strains from India were sequenced to identify their genotypes and sequence variability, and furthermore the data were used to investigate selection/evolution pressure and structure prediction using homology modeling.

2. Materials and methods

2.1. Sample collection and preparation

A total of 23 RVA positive diarrheic faecal samples collected during active surveillance for the presence of enteric viruses within the bovine (cattle and buffalo) population of India from 2008 to 2011, were analyzed in this study (Table 1). The faecal samples were processed by making 10% faecal suspension (w/v) in phosphate buffered saline (0.01 M, pH 7.4; Sigma, USA) followed by centrifugation at 2000×*g* for 10 min and the filtration of upper aqueous layer through 0.22 μm syringe filter (MDI, India). The filtrates were archived and stored at −20 °C until further use.

2.2. Extraction of viral RNA and reverse transcription polymerase chain reaction

The viral RNA extraction and cDNA synthesis were carried out as per the method described earlier (Malik et al., 2012). The full length NSP4 gene (743 bp) was amplified using degenerate primers: (NSP4(1–28)[+] GGCTTTWAAAAGTTCTGTTCCGAGAGAG; NSP4(722–743)[−] TAAGACCRTTCCYTCCATTAAC) and the reaction was performed at initial denaturation at 95 °C for 5 min followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 90 s at 72 °C, and final extension at 72 °C for 5 min. The PCR amplicons were gel purified using the GeneJet Gel Extraction Kit (Fermentas, EU), cloned into the pGEMT Easy Vector cloning system (Promega) and sequencing was outsourced to M/S SciGenom Labs Pvt. Ltd., Kerala, India.

2.3. Sequence and phylogenetic analysis

The NSP4 sequences used in this study ($n = 23$) along with other NSP4 sequences from India and across the world were retrieved from The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Sequences were aligned by ClustalW and a dendrogram was constructed in MEGA5 (Tamura et al., 2011) by Neighbor Joining (NJ) statistical method using the Maximum Composite Likelihood substitution model with 2500 bootstrap replicates. The nucleotide and deduced amino acid sequence identities of NSP4 genes were analysed with NSP4 sequences of rotaviruses from different species and geographical locations published in GenBank database. Furthermore, the RotaC v2.0 web based tool for RVA classification (http://rotac.regatools.be) was used to determine the sequence E genotypes (Maes et al., 2009).

2.4. Selection pressure analysis

Site specific selection pressure was measured on gene segment 10 using the HyPhy software implemented in the Datamonkey web server (Kosakovsky Pond and Frost, 2005a). In this study, a total of 119 NSP4 coding sequences of bovine rotavirus A (96 sequences retrieved from NCBI and 23 sequences of present study) were chosen and this server accepted 80 sequences by declining the similar/duplicate sequences (Supplementary data 1). The sites under selection pressure were evaluated using classic maximum likelihood methods i.e. Single Likelihood Ancestor Counting (SLAC) model and Fixed Effect Likelihood (FEL) model (Kosakovsky Pond and Frost, 2005b) and other more recently developed methods; Mixed Effects Model of Evolution (MEME) capable of identifying

Table 1
Bovine rotavirus NSP4 sequences considered in this study from different geographical regions of India.

S. No.	Rotavirus strains	Place of isolation	RotaC$^{2.0}$ based genotyping	Amplicons size (bp)	GenBank accession Nos.	References
1	RVA/Buffalo-wt/IND/B54/2008/G3P[8]	Haryana	E1	534	HQ433436	Unpublished
2	RVA/Buffalo-wt/IND/B111/2009/GXP[X]	Haryana	E2	554	JF831958	Unpublished
3	RVA/Buffalo-wt/IND/BRV133/2010/G3P[11]	Haryana	E2	568	HQ157345	Unpublished
4	RVA/Cattle-wt/IND/CC156/2010/G3P[X]	Haryana	E2	519	HQ157349	Unpublished
5	RVA/Cattle-wt/IND/B108/2011/GXP[X]	Haryana	E2	500	JX442776	Unpublished
6	RVA/Buffalo-wt/IND/B47/2008/GXP[X]	Madhya Pradesh	E2	724	HM591492	Unpublished
7	RVA/Buffalo-wt/IND/B48/2008/G3P[3]	Madhya Pradesh	E2	724	HM591493	Unpublished
8	RVA/Buffalo-wt/IND/B68/2008/G3P[X]	Madhya Pradesh	E2	739	HQ433435	Unpublished
9	RVA/Buffalo-wt/IND/B72/2008/G10P[1]	Madhya Pradesh	E2	549	HQ157346	Unpublished
10	RVA/Buffalo-wt/IND/B100/2008/G3P[3]	Madhya Pradesh	E2	724	HM591494	Unpublished
11	RVA/Cattle-wt/IND/C51/2008/GXP[X]	Madhya Pradesh	E2	566	HQ157350	Unpublished
12	RVA/Cattle-wt/IND/C1/2010/GXP[X]	Madhya Pradesh	E2	677	JF831956	Unpublished
13	RVA/Buffalo-wt/IND/B212/2010/GXP[X]	Madhya Pradesh	E1	678	JF831954	Unpublished
14	RVA/Cattle-wt/IND/MF10/2010/G3P[3]	Madhya Pradesh	E2	517	JX442768	Unpublished
15	RVA/Cattle-wt/IND/15E/2009/GXP[1]	Uttarakhand	E2	737	HQ171910	Unpublished
16	RVA/Cattle-wt/IND/P9/2009/G3P[1]	Uttarakhand	E1	744	JF689836	Unpublished
17	RVA/Cattle-wt/IND/P14/2009/G3P[1]	Uttarakhand	E2	534	HQ157344	Unpublished
18	RVA/Cattle-wt/IND/P970/2009/G3P[1]	Uttarakhand	E2	568	HQ157348	Unpublished
19	RVA/Cattle-wt/IND/C5/2010/GXP[X]	Uttarakhand	E2	726	JF831947	Unpublished
20	RVA/Cattle-wt/IND/Bov1/2009/G3P[1]	Uttar Pradesh	E2	726	JF689837	Unpublished
21	RVA/Buffalo-wt/IND/Bov2/2009/G3P[1]	Uttar Pradesh	E2	663	JF831952	Unpublished
22	RVA/Buffalo-wt/IND/BE3/2010/G6P[X]	Uttar Pradesh	E2	497	JF831957	Unpublished
23	RVA/Cattle-wt/IND/H6/2011/GXP[X]	West Bengal	E2	573	JX442789	Unpublished