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Rotavirus Nonstructural Protein 4 (NSP4)-Viral Enterotoxin with Multiple roles in Pathogenesis of Diarrhoea in Children

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ABSTRACT

Nonstructural protein 4 (NSP4) of Rotavirus has been designated as the first viral enterotoxin. Its role in viral replication is already well known. Intensive research over the past decade has shown the involvement of this protein in many cellular activities both in 'expressed' as well as 'secreted' form. It is responsible for increased intracellular calcium levels in the infected cell leading to a cascade of events which involve phospholipase C mediated secretion of Chloride ions. NSP4 also inhibits intestinal disaccharidases and sodium glucose symporter (SGLT1) so that complex sugars are retained resulting in malabsorption. It also alters actin in the villi resulting in their flattening and overall decreased absorptive area. NSP4 is associated with extracellular proteins and is hypothesized to have paracrine effects on neighbouring cells. Recent research has found it to be an activator of enteric nervous system too. All these factors contribute to the pathogenesis of diarrhoea which looks multifactorial and certainly very different from the bacterial toxin mediated diarrhoeas of E. coli and V. cholerae. We still don't have the final word on this intriguing protein which is now a potential candidate for a vaccine against rotavirus. The aim of this review is to put forward the salient features of the research done to elucidate the functions of NSP4.

INTRODUCTION

Infectious acute diarrhoea causes considerable morbidity and mortality in infants and children less than five years of age. Bacterial agents of diarrhoea and their pathogenesis was elucidated in the first half of this century but it was not until the 1970s with the discovery of Norwalk virus by Kapikian *et al.*, (1972) and subsequently of Rotaviruses by Bishop *et al.*, (1973) that a headway was made regarding the viral etiology of diarrhoea. Since then Rotaviruses have been established as the most important viral cause of diarrhoea in the young of both humans and animals. Worldwide 611,000 deaths annually are attributed to rotavirus infection only. (Parashar *et al.*, 2006) Rotavirus is a double stranded RNA virus belonging to the family Reoviridae. Mature viral particles are enveloped, 100 nm in diameter with icosahedral symmetry. A triple layered capsid surrounds a genome of 11 segments of double stranded RNA. The genome codes for six structural proteins VP1,VP2,VP3, VP4,VP6 & VP7 and six non-structural proteins NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6. VP7 and VP4 constitute the outer most layer; VP6 forms the intermediate layer and VP2 is present in the innermost layer (Figure 2).

Differences in the antigen specific epitopes on the protein VP6 are the basis of classification of rotavirus into 7 groups (A-G). Group A rotavirus are the most frequent pathogens of humans and groups D-E have been found only in animals.

Group B rotaviruses have caused epidemics affecting only adults in China while group C is a relatively rare cause of diarrhoea in children. *We are referring only to group A rotaviruses in this review.*

Rotaviruses exhibit two distinct serological specificities namely subgroup and serotype. Subgroup I, II, I+II and non-I/ II are based on the different antigens of VP6. VP7 and VP4 possess neutralization antigens and form the basis of the current dual classification of group A rotavirus into G and P serotypes. (Estes & Kapikian, 2006)

Initially Non structural protein 4 (NSP4) was thought to play a role primarily in viral replication. But recently it has generated a lot of attention in viral research on being designated as the "first viral enterotoxin" and the major virulence factor responsible for the watery diarrhoea of rota virus infection.

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The underlying mechanisms have since been extensively researched and NSP4 has been found to have multiple effects on cell permeability, intracellular calcium levels, Sodium – glucose symporter (SGLT1), fibronectin and laminin among other parameters investigated in various study designs.

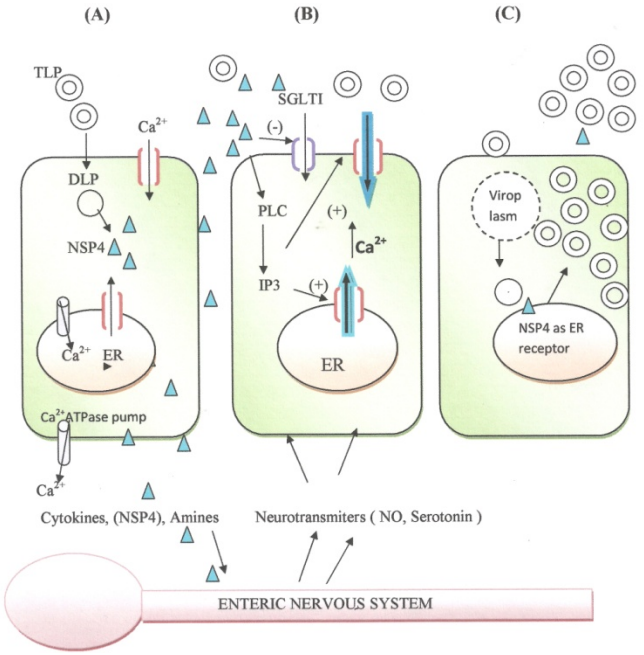

Fig. 1:
A: EARLY INFECTION OF MATURE ENTEROCYTE – Expression of NSP4 and secretion from basolateral and apical sides. Cytosolic free Ca^{2+} levels (Ca2+) are regulated by two main mechanisms working in opposite directions. First are the Ca^{2+}-ATPase pumps which actively push out Ca^{2+} from cytoplasm to either the ECF or to the intracellular compartments such as ER and second are the Ca^{2+}channels on the ER and cytoplasmic membrane which allow passive flow of Ca^{2+} inside the cell.
B: LATE INFECTION - NSP4 interacts with a receptor in the gut epithelium and stimulates a calcium dependent signal transduction pathway involving phospholipase C which results in efflux of chloride ions with attendant water molecules across the plasma membrane. NSP4 also has an inhibitory effect on SGLTI leading to malabsorption component of diarrhoea
C**: VIRAL REPLICATION AND CELL LYSIS** – NSP4 as ER resident receptor for DLP budding inside ER and final assembly into TLPs
ER = Endoplasmic reticulum; **TLP** = Triple layered particle; **DLP** = double layered particle; **SGLTI** = Sodium glucose symporter**; PLC** = Phospholipase C**; IP3** = Inositol-3-phosphate

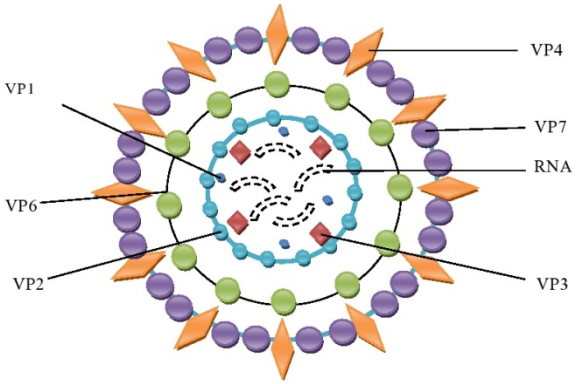

Fig. 2: Structure of rotavirus virion.
Six structural proteins of rotavirus form the triple layered capsid. VP7 and VP4 form the outer shell; VP6 forms the middle layer and VP2 forms the innermost layer. VP = Viral protein.

NSP4- MORPHOLOGY AND STRUCTURE

Non structural protein 4 (NSP4), encoded by segment 10 is composed of 175 amino acids. The primary 20 kDa polypeptide is cotranslationally glycolysated to 29 kDa which is further processed to a 28 kDa protein. The amino terminal region of the protein contains an uncleaved signal sequence and three hydrophobic domains H1, H2, and H3. H1 domain contains two N- linked high mannose glycolysation sites and most of the residues float within the endoplasmic reticulum(ER) lumen. H2 is sandwiched within the ER membrane. H3 and rest of the residues upto the C terminus of the protein are hydrophilic and reside in the cytoplasm (Both *et al.*, 1983; Chan *et al.*, 1988 and Bergmann *et al.*, 1989). Various functions have been attributed to different domains of the protein. The region between amino acid (aa) 55 and 72 has been found to possess plasma membrane destabilizing activity (Newton *et al.*, 1997) while aa between 114-135 after cleavage from the parent protein has enterotoxic properties. The VP6, VP4, and tubulin binding sites correspond to NSP4 residues 161 to 175, 112 to 148, and 129 to 175, respectively. The C-terminus has been shown to bind double layered viral particles (DLPs). NSP4 residues 95 to 137 correspond to a oligomerization domain that has amphipathic properties (Figure 3).

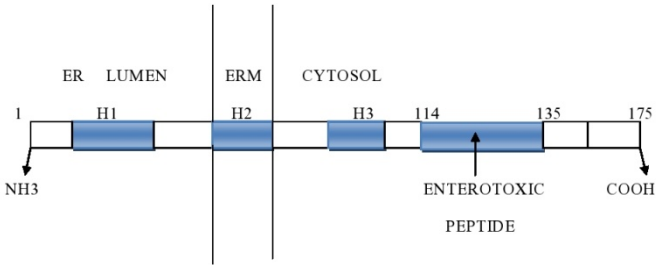

Fig. 3: Structural organization of NSP4 protein.
H1 domain of amino terminal lies in the cytosol, H2 within the endoplasmic membrane and H3 and rest of the carboxy terminal within the lumen of endoplasmic reticulum. The peptide from amino acid position114 – 135 has enterotoxic properties.
H1=Hydrophobic domain 1; H2= Hydrophobic domain 2; H3 = Hydrophobic domain 3; ER=endoplasmic reticulum; ERM=endoplasmic reticulum membrane.

ROLE OF NSP4 IN VIRAL REPLICATION AND ASSEMBLY

Rotaviruses being gastrointestinal pathogens the target of viral entry and replication was believed to be the differentiated enterocyte. Recent reports about extra intestinal spread of infection has changed this perception and other cells may be involved at some stage or other. The exact mechanisms of viral entry and uncoating are incompletely understood. Replication takes place in the cytoplasm with the ER playing a crucial role. As the double layered virus particles (DLPs) devoid of the outer layer composed of VP4 and VP7 are formed they start aggregating as 'viroplasms' in the cytoplasm.

Subsequently, DLPs bud into the ER through the interaction between VP6 and C terminal of NSP4, which acts as an ER resident receptor to transfer the viroplasm to the ER lumen (Au

et al., 1993). During the budding process, the virion acquires VP7 and a temporary envelope. Once inside the ER, VP4 joins VP7 to complete the outer layer. The lipid envelope and NSP4 are shed by a process yet to be deciphered. Mature virions have no structural proteins and stay for a while in the ER before being finally released by cell-lysis (Taylor *et al.,* 1992; Taylor *et al.,* 1993).

Cells in which NSP4 expression has been stalled by using small interfering RNAs, viroplasm formation is inhibited as expected but additionally cellular distribution of some other proteins is also affected. Viral m RNA synthesis also seems to be altered (Lopez *et al.,* 2005; Silvestri *et al.,* 2005). Overall the role of NSP4 in replication needs to be researched further so that gaps in the existing knowledge could be filled.

SECRETION OF NSP4 PEPTIDE AND INTERACTION WITH EXTRACELLULAR MATRIX PROTEINS

The mature virion does not contain NSP4 or any of the other structural proteins. During active infection of the enterocyte NSP4 is translated and cleaved and a peptide (112 to 175) is actively secreted from the apical side. This peptide was hypothesized to have paracrine effects on the nearby cells (Zhang *et al.,* 2000).

Other workers showed that NSP4 is released from the basal side of the infected epithelial cells also. They were able to demonstrate physiological interactions of NSP4 with laminin β -3 and fibronectin which are basically multifunctional proteins present as major constituents of basement membranes and thought to play a vital role in the assembly of extra cellular matrix. This was a very significant finding as till that time it was widely believed that NSP4 (aa 112 to 175) is actively secreted only from the apical side of infected epithelial cells via an atypical pathway that bypasses the Golgi apparatus and involves lipid microdomains called rafts (Jourdan *et al.,*1997; Sapin *et al.,* 2002). After release from the apical side it was thought that it binds to a receptor and directly or indirectly triggers a signal transduction pathway to enhance chloride secretion, eventually resulting in diarrhea (Ball *et al.,* 1996; Morris *et al.,* 2001). This secretory mechanism, however, was proposed to occur in the intestinal crypts (Lundgren *et al.,* 2000). The intestinal crypts have a continuous flow of secretions against which diffusion of NSP4 did not seem plausible.

The findings of Boshuizen *et al.,* led to speculations that NSP4 travels towards the crypt region where it can induce fluid secretion via a route involving the ECM. Further findings by Ball and coworkers that NSP4 is able to cause chloride secretion when added to the submucosal (basal) surface of mouse mucosal sheets support the above view. It was also found that secreted NSP4 stays soluble in an aqueous environment as an oligomeric lipoprotein that has the property of binding to various cell types via an interaction with glycosaminoglycans . This broad cellular tropism exhibited by NSP4 is supported by findings that indicate that rotavirus infection is not confined to the intestine but instead leaves the intestine and enters the circulatory system (Blutt *et al*., 2003; Mossel *et al.,* 2003). Rotavirus has been demonstrated in cerebral spinal fluid (Nishimura *et al.,* 1993), liver and kidney, extraintestinal lymphoid tissue (Brown *et al.,* 1998) and even in the heart, where it was mainly localized to endothelial cells (Mossel and Ramig 2003).

It has been proposed that rotavirus, enters the circulatory system through M cells that are present in the epithelium overlying Peyer's patches (Blutt *et al.,* 2003). Above findings also imply that NSP4 could get into the circulatory system, after being secreted into the extracellular matrix underlying the enterocytes. .

Localization of NSP4 within the cell.

NSP4 seems to have three different addresses within the same cell depending on the level of expression and the time since infection. The first address is the ER where it can be found throughout the infection. Second place is the ER Golgi Intermediate compartment (ERGIC) where the cleavage and delivery of the peptide (114- 135) takes place through a non classical pathway. A third address is in the cytoplasm within vesicular structures tagged with the autophagosomal marker LC3. Here NSP4 can be found as early as 6 hours after infection and is believed to recruit these vesicles to viroplasms. In all probability this association prevents fusion of lysosomes with viroplasms there by extending their lifespan and overall enhanced viral replication. Further research of the autophagy pathway are needed to confirm the above hypothesis (Berkova *et al.,* 2003)

ASSOCIATION OF NSP4 WITH CAVEOLAE AND CELLULAR SIGNALLING

Parr *et al.,* (2005) have shown that both NSP4 and the enterotoxic peptide (NSP4 114-135) show predilection for caveola-like model membranes. Caveolae are a type of lipid rafts that are rich in cholesterol and sphingomyelin and are found in most cells, including enterocytes. They contain the integral membrane protein caveolin-1 which functions mainly as a platform to organize signaling molecules at the plasma membrane. The apparatus of Ca^{2+} regulation and signaling consisting of Ca^{2+}ATPase, inositol trisphosphate receptors, tyrosine kinases, G proteins, and calmodulin is found in caveolae. In addition, caveolae are home to as much as 50% of cellular Phosphatidylinositol diphosphate (PIP2) and are the site of PIP2 hydrolysis in response to a ligand (Pike *et al.,* 1996). The above authors demonstrated colocalization of NSP4 and caveolin-1 in NSP4-transfected and rotavirus-infected mamalian cells in ER, in the cytoplasm, and at the cell membrane using laser scanning confocal microscopy and proposed that the association of NSP4 and caveolin-1 contributes to NSP4 intracellular trafficking from the ER to the cell surface.

The binding site for caveolin-1 has been demonstrated in the hydrophobic region of the amphipathic helix (Ball *et al.,* 2013). These findings also suggest that NSP4 can have a role in cellular signalling as discussed in the following sections.

ROLE OF NSP4 AS THE VIRAL ENTEROTOXIN AND PATHOGENESIS OF DIARRHOEA

Rotavirus infects the mature enterocytes in the mid and upper villous epithelium of the small intestine, which ultimately leads to cell death, villous atrophy, and diarrhea. Many workers have put forward various theories regarding the causative mechanism of diarrhoea. These include enterocyte death , villus ischemia Starkey *et al.*, 1986) , a toxin-like effect of the non-structural protein (NSP4) and activation of the enteric nervous system resulting in electrolyte and fluid secretion (Lundgren *et al*., 2000). Experiments in which NSP4 was administered intraperitonealy in infant mice resulted in diarrhoea in an age dependent manner (Ball *et al.*, 1996; Horie *et al.*, 1999). Ball *et al.*, (2000) also demonstrated that a peptide sequence from aa 114 to135 (crosslinked) of the 175 amino acid protein from SA11 strain was specifically responsible for diarrhoea in the mouse model. Another important finding was that diarrhoea associated with the early stages of rotavirus infection appeared to be mediated by enhanced fluid transport before gross histological changes in the mucosa occur.

In another study neonatal pigs inoculated with porcine rotavirus developed watery diarrhoea 8 h after infection, whereas a few histopathological lesions were found in jejunum segments after nearly two days of infection (Vellenga *et al.*, 1992). Rotavirus-infected mouse cells when scanned under electron microscope were found to contain lots of swollen ER cisternae and vacuoles beneath the apical membrane (Starkey *et al.*, 1986) but as such no significant gross morphological changes within the gastrointestinal lining were observed. These studies demonstrated that rotavirus-induced diarrhoea is not as a result of gross structural damages and disruption of microvilli leading to malabsorption as initially believed. As it was observed that induction of diarrhea and alterations in chloride secretion occurred within a period of about 3 -8 h, homologous to those induced by the heat-stable toxin b of *Escherichia coli*, NSP4 was proposed to be a viral enteotoxin.

The cascade of events leading to fluid secretion has been intensively researched and many crucial mechanisms have been deciphered. The basic theory that has been proposed is that NSP4 interacts with a receptor in the cells lining the GIT and sets in motion a signal transduction pathway involving phospholipase C which results in exit of chloride ions aswell as water molecules across the plasma membrane. The resulting secretory diarrhoea was found to be calcium dependent (Ball *et al.*, 1996 and Zhang *et al.*, 2000).

Status of Calcium and interplay with NSP4

NSP4, when added 'exogenously' as well as when 'expressed' intracellularly, is supposed to be a key player in intracellular calcium regulation during rotavirus infection. Historically calcium levels have been shown to be vital for replication. In cells with depleted calcium, viral maturation and assembly was stalled after the DLPs enter the ER (Shahrabdi and Lee., 1986). Intracytoplasmic calcium ions (Ca^{2+}) are regulated by two main mechanisms working in opposite directions. First are the Ca^{2+}-ATPase pumps which actively push out Ca^{2+} from cytoplasm to either the ECF or to the intracellular compartments such as ER and second are the Ca^{2+}channels on the ER and cytoplasmic membrane which allow passive flow of Ca^{2+} inside the cell. (Figure 4)

Michelangeli *et al.*, (1991) reported that rotavirus protein synthesis in MA104 cells leads to elevated intracellular calcium levels which they proposed was as a result of increased permeability of plasma membrane.

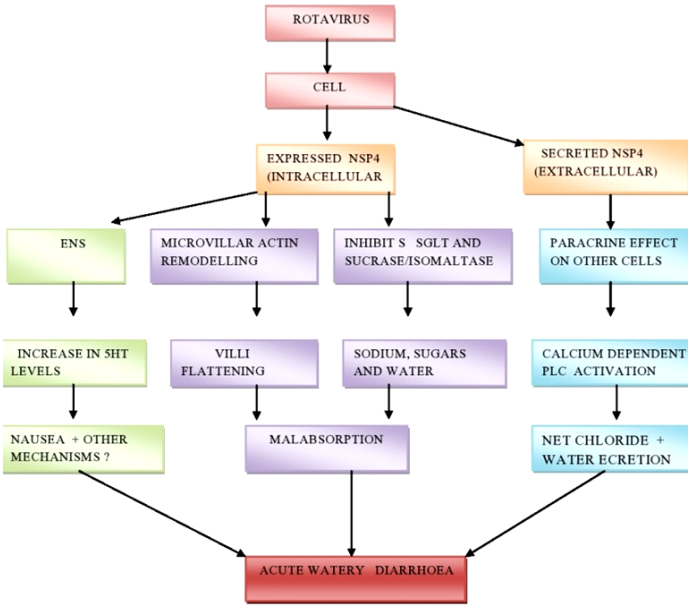

Fig. 4: Theories of diarrhoeal pathogenesis
NSP4 = Nonstructural protein 4; ENS = Enteric nervous system; PLC = Phospholipase C; 5HT = 5 Hydroxy tryptamine; SGLT1 = NA+-D-glucose symporter

It was observed that 'expression' of NSP4 is responsible for an increase in (Ca2+) in *Spodoptera frugiperda* (Sf9) insect cells infected with baculovirus expressing the NSP4 gene (Tian *et al.*, 1994). In another study the same authors investigated the underlying pathophysiology and measured the permeability of both plasma and ER membrane in the same setting and found that though NSP4 expression increases the basal Ca^{2+}permeability of the ER membrane no significant changes were found in permeability of plasma membrane as previously proposed by Michelangeli *et al.* They also added purified NSP4 protein or a 22-amino-acid synthetic peptide consisting of residues 114 to 135 (NSP4 114–135) to noninfected Sf9 cells to assess the paracrine effects of secreted NSP4 on [$Ca2^{2+}$]i. Both NSP4 and the NSP4 [114–135] peptide produced a time-dependent increase in (Ca2+) that was reduced if phospholipase C was blocked with its inhibitor U-73122.Also in the same cells if ER Ca ATPase was inactivated with thapsigargin the increase in (Ca2+) produced by NSP4 114–135 was completely abolished, but the peptide only partially reduced the change in (Ca2+) produced by thapsigargin. No changes in (Ca2+) were seen in cells treated with control peptides

(Tian *et al.,* 1995). Other workers monitored (Ca^{2+}) in human colonoic adenocarcinoma cell line [HT-29] using microscope-based fluorescence imaging. NSP4 induced both Ca^{2+}release from intracellular stores and plasmalemma Ca^{2+}influx. During NSP4-induced (Ca2+) mobilization, [Na^{+}]i homeostasis was not affected, demonstrating that NSP4 selectively regulated extracellular Ca^{2+}entry into these cells. Pretreatment of cells with either trypsin or chymotrypsin for 1–10 min terminated the NSP4-induced (Ca2+) mobilization. U-73122 again ablated the NSP4 response. NSP4 also induced a rapid onset and transient stimulation of inositol 1, 4, 5-trisphosphate (IP3) production in an IP3-specific radioreceptor assay (Dong *et al.,* 1997). Human intestinal epithelial cell line Caco-2 was infected by RRV strain in experiments done by Brunet *et al* (1999).They reported that viral replication induces (Ca^{2+}) increase through an altered Ca^{2+} permeability of plasmalemma in the initial phase of infection and also by a PLC-dependent efflux of Ca^{2+} from the ER at late stage of infection.

To summarize ' expression' of NSP4 increases (Ca^{2+}) in early phase of infection due to increased permeability of both cytoplasmic and ER membranes. As no effect of U-73122 was seen on the elevated (Ca^{2+}) in cells expressing NSP4, phospholipase C does not appear to play any significant role in this initial phase. Later on as NSP4 accumulates in the secreted form , it increases(Ca^{2+}) by primarily involving receptor- mediated phospholipase C activation and IP3 production.. As discussed the peptide length 114- 135 is the region critical for this activity.

Effect on membrane permeability

As discussed above NSP4 and NSP4 (114-135) produce significant changes in the permeability of cellular membranes specially those of the ER resulting in raised (Ca^{2+}). In another study expression of NSP4 in monkey kidney epithelial cells resulted in loss of plasma membrane integrity, which could be demonstrated by release of lactate dehydrogenase into the medium. The cytotoxic activity of NSP4 was proportional to the amount expressed. Morphological analysis revealed gross changes to cell ultrastructure, indicative of cell death. Additional analysis of NSP4 deletion mutants suggested that a region located within the cytoplasmic part of the protein may mediate cytotoxicity (Newton *et al.,* 1997)

Another theory about the 'secretory component' of rotavirus diarrhoea was that may be NSP4 has some sort of a direct effect on the Chloride transport across the intestinal epithelium.

The direct effect of the rotavirus NSP4 114-135 peptide on Cl uptake was demonstrated in experiments on villus cell brush border membrane (BBM) derived from rabbit newborns. The peptide inhibited the Cl^{-}/H^{+} symport activity in a nonspecific manner. The interaction involved only one peptide-binding site per carrier unit. Thus there is lab in vito evidence of NSP4 (114-135) being involved in chloride transport. To assess the situation in vivo infant rabbits were exposed to the peptide and it was seen that both Cl^{-} absrption and Cl^{-} secretion across villi BBM were enhanced without any change in Cl^{-} transport in crypt BBM and ultimately there was no direct, specific effect on either intestinal absorption or secretion of chloride. The lack of direct effect of NSP4 on chloride transport also strengthens the hypothesis that NSP4 most probably involves signal transduction pathways to produce net chloride secretion at the onset of rotavirus diarrhoea (Lorrot *et al.,* 2006). In a recent study chloride secretion induced by NSP4 was found to be related to increase in oxidative stress (Buccigrossi *et al.,* 2014).

Effect on microvillar actin; intestinal enzymes & Na^{+}-D-glucose symporter (SGLTI) -Malabsorption component of diarrhoea?

Like many other pathogens, rotavirus infection and replication leads to rearrangement of the cytoskeleton with disorganization of cytoskel *et al* elements such as actin and cytokeratin. Brunet *et al.,* (2000) have demonstrated that in polarized human intestinal Caco-2 cells line, rotavirus infection induces calcium-dependent depolymerization of microvillar actin leading to disruption and flattening of microvilli as observed under electron microscopy. The immediate effect is that the surface area for absorption is drastically reduced resulting in malabsorption. In the same study it was also shown that a viral protein secreted into the medium had the same effects on microvillar actin in noninfected cells. The candidature of NSP4 as a likely player was proposed and further research showed that expression of NSP4, caused stabilization of long cellular projections in HEK 293 cell line. Quantification of filamentous actin (F-actin) content revealed an elevated F-actin content in NSP4 expressing and rotavirus-infected cells as opposed to that in nonexpressing and noninfected cells. Intracellular calcium levels were also vital for these changes in F-actin content as was an increased activation of the actin remodeling protein cofilin. Another point to notice is that actin framework of a cell is responsible for activities such as transport of ions, cell division and release of viral progeny and these activities could also be affected (Berkova *et al.,* 2007).

Rotavirus infection in the mouse model has been found to decrease the activity of intestinal disaccharidases without any appreciable effect on the intestinal brush border membrane (Collins *et al.,* 1990). Among the four brush border disaccharidases expressed in the small intestine, Sucrase isomaltase (SI), a enzyme complex which hydrolyzes maltose, maltotriose, and sucrose, has been extensively studied in vivo as well as in the enterocyte-like model Caco-2 cell line (Hauri *et al.,* 1988). Jourdan *et al.,* (1998) reported that both the activity and expression of SI *in vitro* in human Caco-2 cells were reduced by rotavirus infection without any histopathological lesions. They were also able to demonstrate that RRV infection did not affect SI production and tertiary structure but interfered with the enzyme's delivery to the brush border membrane. Another evidence collected was that of Rotavirus infection inducing an important alteration of the brush border-associated cytoskeleton that correlates with decreased SI apical surface expression. As previously discussed NSP4 has already been implicated in inducing changes in cellular cytoskeleton (Berkova *et al.,* 2007).

Rotavirus infection has a negative effect on NA+-D-glucose symport (SGLTI) activity in both villus and crypt cell BBM of rabbit intestine. The active resorption of glucose was found to be higher for villi BBM in infected rabbits than for crypt cell BBM in control rabbits not exposed to rotavirus. There was no effect on SGLT1 protein expression, which was higher for villi than for crypt cells. This was an interesting finding as it became clear that enterocytes could not be crypt-like cells as proposed by a few workers in the crypt-cell invasion theory (Lorrot *et al.*, 2006 ; Halaihel *et al.*, 2000) In further experiments done on rabbit intestinal BBM, the NSP4-(114–135) peptide has been shown to directly and strongly inhibit SGLT1, but not Na+-L-leucine symport activities (Halaihel *et al.*, 2000). To summarize NSP4 is definitely involved in glucose malabsorption during rotavirus infection *in vivo*. By inducing maldigestion of carbohydrates and their accumulation in the intestinal lumen as well as malabsorption of nutrients and a simultaneous inhibition of water reabsorption, which can lead to malabsorption component of diarrhea

Effect on enteric nervous system and enterochromaffin cells

The research during the past decades has led to the observation that intestinal secretions induced by bacterial pathogens are partially due to activation of enteric secretomotor neurones. The same has been suspected for rotaviral gastroenteritis too. The enteic nervous system (ENS) consists of two layers within the intestinal wall, the myenteric plexus and the submucosal plexus. The functions are intertwined but the myenteric layer is more involved with muscle control and the submucosal with managing secretion. ENS is relatively huge given that it consists of 100 million neurons which are almost equal to those of spinal cord. Like the CNS, the ENS releases neurotransmitters such as serotonin, acetylcholine, and nitric oxide. and dopamine. Out of these serotonin is implicated to be the most important neurotransmitter. Stimulation of 5HT3 and 5HT4 receptors initiates peristalsis and facilitates secretion in the gastrointestinal tract (GIT) Particularly rich in serotonin are the enterochromaffin cells of the GIT mucosa which are present in the middle and upper segments of villi in the duodenum, jejunum and ileum and are classically associated with rotavirus replication and histopathological lesions. Enterochromaffin cells release mediators of endocrine signalling from the basolateral surface to activate afferent neuron endings within the lamina propria. They also appear to be sensitive to mechanical stimulation and may be involved in the peristaltic reflexes of the GIT (Bertand *et al.*, 2000; Hansen and White, 2008). Kordasti *et al.*, (2004) demonstrated that rotavirus infection results in stimulation of the ENS. They were also able to demonstrate that rotavirus diarrhoea in mice can be attenuated with 5-HT3 receptor antagonists such as granisetron. Other researchers showed that crude and virus particle-free supernatants from rotavirus-infected MA104 cells stimulated 5-HT release within an hour in primary EC cells and carcinoid EC cell line (GOT1). Again recombinant and secretory NSP4were able to release 5-HT from primary and GOT1 EC cell line (Hagborn *et al.*, 2011).

Molecular epidemiology association with virulence

After sequencing of NSP4 gene from both human and animal strains and subsequent analysis five genotypes (A-F) were assigned initially. The Rotavirus Classification Working Group (RCWG) has assigned a total of 14 NSP4 genotypes (E1-E14)(Matthijnssens *et al.*, 2008, 2011). As molecular sequencing database expands the number may rise in the future. The most common NSP4 genotypes in humans are E1 (Wa-like), E2 (Kun-like) and E3 (AU-1), which up till now were known as genotypes B, A and C respectively. Some other uncommon genotypes like E6 and E9 have also been reported from a few geographical areas (Khamrin *et al.*, 2007; Rehman *et al.*, 2007).

In one study mutation causing amino acid substitutions in the region between 130 -141 aminoacid from two pairs of virulent and attenuated (tissue culture adapted) swine strains were found to have altered rotavirus virulence in mice (Zhang *et al.*, 1998).

IMMUNE RESPONSE TO NSP4

Historically structural proteins VP4 and VP7 induced formation of type specific neutralizing antibodies has been considered to confer protection though the correlates for protection have not been clinically demonstrated. Studies on hepatitis C virus and some flaviviruses have shown that non structural proteins can induce protective immunity. In children suffering from non rotavirus diarrhoea antibodies against NSP4 were demonstrated in acute phase serum and may have conferred protection from rotavirus (Vizzi et al., 2005). Several workers have studied humoral immune response against NSP4 in detail. In human infants vaccinated with a live oral vaccine a good IgG and IgA response against NSP4 has been obtained which is not type specific or homotypic (Yuan *et al.*, 2004). Ray *et al.*, (2003) have reported significant heterotypic humoral response to NSP4 in a study done in children suffering from rotavirus gastroenteritis

In another study gnotobiotic calves and piglets were first infected with bovine NSP4 A or porcine B genotype and subsequently challenged with recombinant NSP4 of various genotypes and derived from different animal and human strains. It was found that antibody production was more if the NSP4 was derived from the same species and also showed an association with species specific region mapping to aa131-141 .The genotype of NSP4 did not make any difference to the overall response (Yuan et al., 2005). Orally given vaccines based on NSP4 have been found to confer protection in mice model (Choi *et al.*, 2006). There are no studies in humans as of now but are surely to follow suit.

CONCLUSION

To summarize research on the functions of NSP4 has been exhaustive to say the least. Regarding the enterotoxin function, there is agreement on a lot of hypotheses put forward by workers but gaps still remain in the final picture which is not as complete as that of bacterial enterotoxins. Various mechanisms are known but the exact contribution of secretory component vs the enteric nervous system vs malabsorption is not clear. What is clear

is the omnipresent association of NSP4 in various mechanisms investigated up till now. The immune reponse against this protein is also being studied.

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