Chapter 2
Review of literature

2.1. History
Looking back into the history, rotavirus was first described in murine species as the agent responsible for “epizootic diarrhea of infant mice” i.e. EDIM (Adams and Kraft, 1963). In the same year viruses of comparable morphologic features were observed in rectal swab specimen of monkeys (Malherbe and Harwin, 1963). These agents were described to have wheel like appearance with 70 nm diameter and hence were later designated “rota” viruses from Latin word for wheel (Flewett et al., 1974). In 1969, Mebus and his coworkers (1969) demonstrated the presence of these particles in faeces of calves with diarrhea, thus associating them with diarrheal disease of cattle. Human rotaviruses were first reported in 1973 by Bishop et al., (1973) in the biopsy specimens of duodenal mucosa from children with acute gastroenteritis. Within a short span of time, these and other investigators confirmed the association between the presence of rotavirus in feces and acute gastroenteritis.

The perception of rotavirus as a major killer dates back to 1985, when the United States Institute of Medicine concluded that 870,000 deaths occurring annually among children below 5 years of age were attributable to rotavirus gastroenteritis (Vesikari T, 2008). This estimate was made at a time when total childhood deaths from diarrheal diseases were estimated to be 4.5 million (Synder and Merson, 1982). With the introduction of oral rehydration therapy (ORT) and other therapeutic advances, the number of diarrheal deaths worldwide now is well under 2 million, whereas deaths due to rotavirus are still estimated between 440,000 and 600,000, indicating rotavirus as one of the most important causative organisms of childhood diseases (Parashar et al., 2003).

2.2. Structure and genome
Rotaviruses are non-enveloped viruses with complex architecture. The virion constitutes a triple layered icosahedral protein capsid and a genome made up of 11 segments of double stranded RNA (dsRNA) coding for 6 structural (VP1-4, VP6 and VP7) and 6 non-structural (NSP1-6) proteins (Figure 2.1) (Estes 1989; Shaw et al., 1993; Mattion et al., 1994; Prasad and Chiu 1994). The outer capsid layer consists of 780 molecules of VP7 glycoprotein arranged around 132
aqueous pores and 60 dimers of VP4 protein which forms spike like projections on the surface of the virion. The VP4 protein is anchored to the intermediate layer composed of 780 molecules of VP6 protein. The innermost layer (viral core) is made up of 120 molecules of shell protein VP2 that interacts with 12 molecules of each of the viral enzymes (VP1, RNA polymerase; VP3, guanylyl transferase) and encloses the 11 segments of dsRNA. The sizes of the genome segments range from 660 to 3300 base pair (bp) and their encoded proteins have molecular weight of 20 to 125 kDa. The virion also possesses three types (I, II, III) aqueous channels that connect the central genome containing part of the virion with the viral surface. These channels are thought to be important in the entry of metabolites (purine and pyrimidine bases, phosphate molecules, ribose sugar) required for RNA transcription and release of newly synthesized mRNA transcripts (Prasad et al., 1988).

The rotavirus genome can be extracted and precipitated into 11 distinct bands by polyacrylamide gel electrophoresis. Rotavirus carries group and genotype specific RNA profile, a property that has been extensively used in epidemiological studies. The characteristic RNA electrophoretic migration of group A rotaviruses commonly detected in human infections follows a pattern of four high molecular weight segments (1 to 4), two middle sized segments (5 and 6), a distinctive triplet of segments (7-9) and two smaller segments (10 and 11).

![Figure 2.I Three-dimensional structure of rotavirus and gene coding assignments.](http://www.brown.edu/Courses/Bio_160/Projects2004/rotavirus/Virology.htm)
2.3. Physicochemical properties

Structurally, rotaviruses are stable particles and the calcium ions present in the outer capsid protein VP7 provides stability to the virions (Ruiz et al., 1996; Gajarado et al., 1997). Treatment of Triple layered rotavirus particles (TLPs) with calcium chelating agents (EDTA and EGTA) removes the outer capsid and results in loss of infectivity (Bridger et al., 1976; Estes et al., 1979). Upon further treatment of double layered particles (DLPs) with chaotropic agents such as sodium thiocyanate or high concentration of calcium chloride, single layered core particles (SLPs) are produced (Almeida et al., 1979; Cohen et al., 1979). The three different rotavirus particles, TLPs, DLPs and SLPs can be separated by gradient centrifugation. In CsCl₂, TLPs have a density of 1.36 gm/cm² and sediment at 520S to 530S, whereas DLPs have a density of 1.38 gm/cm² and sediment at 380S to 400S (Tam et al., 1976). Likewise, SLPs have a density of 1.44 gm/cm² in CsCl₂ with a sedimentation coefficient of 280S.

Rotaviruses are relatively resistant to environmental inactivation and survive well in porous (paper, cotton cloth) and nonporous (aluminum, china clay, latex) surfaces of environment (Abad et al., 1994). Infectivity is stable within pH range 3.0 to 9.0 and at +4°C or +20°C for months in presence of 1.5 mM CaCl₂ (Welch and Thompson, 1973; Palmer et al., 1977; Estes et al., 1979; Weiss and Clark, 1985). Particle integrity and infectivity are generally resistant to fluorocarbon extraction and exposure to ether, chloroform and deoxycholate (Fernelius et al., 1972; Welch and Thompson, 1973; Tam et al., 1976; Estes et al., 1979). Sodium dodecyle sulfate (1%) inactivates infectivity, however exposure to non ionic detergents can enhance infectivity, probably by disrupting aggregates (Ward and Ashley, 1980). Disinfectants such as, ethanol, phenol, formalin, chlorine and β-propiolactone inactivate rotavirus by removing the outer capsid (Bishai et al., 1978; Brade et al., 1981; Sattar et al.,1983).

2.4. Coding assignments and proteins

The coding assignments and many characteristics of the rotavirus proteins encoded in each of the 11 segments of genome are well established, even though new functions of the proteins continue to be unveiled. The nomenclature of the structural and non structural proteins is designated as VP (viral protein) and NSP respectively followed by a number depending on the molecular weight (viz. VP1 with highest mol wt. to VP8 with lowest mol wt, likewise NSP1 with
highest mol wt. to NSP6 with lowest mol wt). The structural proteins are present in the virus particles while the non structural proteins are there in the infected cells.

The major structural proteins

The major structural proteins (VP4, VP6 and VP7) of rotavirus have been studied extensively because of their unique biochemical, antigenic and biologic roles in replication and assembly.

The VP4 protein

VP4 is the non glycosylated protein product of gene segment 4. It is a protease sensitive (P) protein present on the outer capsid as a series of spikes approximately 10-12 nm in length, with a knob like structure at the distal end (Shaw et al., 1993). VP4 constitutes 1.5% of the virion protein and has a molecular weight (MW) of 88 kilo Dalton (kDa). It functions as the viral attachment protein in-vitro and in-vivo and is a determinant of viral growth in vitro and a virulence factor in vivo (Hoshino et al., 1995; Bridger et al., 1998).

Proteolytic cleavage of VP4 protein into VP5 (60 kDa) and VP8 (28 kDa) results in enhancement of viral infectivity (Estes et al., 1981). VP8 is responsible for sialic acid dependent binding to host cell and has haemagglutinin activity in many animal rotavirus strains. VP5 functions as sialic acid independent attachment protein and increases membrane permeability to facilitate virus entry (Denisova et al., 1999). VP8 contains greatest sequence variation in VP4 and determines P genotypes. VP4 induces neutralizing antibodies (NAbs) which neutralize virus by inhibiting attachment as well as entry into host cell in vitro and passively protect mice against homologous and heterologous rotavirus challenge in-vivo (Kitaoka et al., 1986; Offit et al., 1986; Burns et al., 1988). Type specific NAbs induced by VP4 have been mapped to VP8 protein while the cross reactive NAbs have been predominantly mapped to VP5 (Larralde et al., 1991).

The VP7 protein

The VP7 is an outer capsid glycoprotein (G) encoded by gene segment 7, 8, and 9 depending on the viral strain. It has a MW of 38 kDa and constitutes 38% of the virion protein (Mattion et al., 1994). It is the second most abundant rotavirus protein that makes basis for G type classification. The native viral VP7 is highly immunogenic protein and induces both type specific and cross reactive NAbs directed to three antigenic regions designated as A, B and C in HRVs
(Dyall et al., 1986). The antigenicity of VP7 is influenced by the N-linked glycosylation sites (Caust et al., 1987).

**The VP6 protein**

VP6 is the major component of rotavirus structure constituting 51% of the virion protein. It is the sixth rotavirus gene product and has a MW of 41 kDa. Naturally it occurs as a trimer and forms the middle layer of triple layered concentric protein capsid coat (Lopez et al., 1994). VP6 plays a key role in the morphology of the virion and act as a physical adaptor between cell entry (outer layer, VP4 and VP7) and RNA packaging (Inner layer, VP2) (Mathieu et al., 2001). Being hydrophobic in nature the VP6 protein is highly antigenic and carries group and sub group epitope specificities of rotavirus measured by various immunoassays. It is highly immunogenic as well and immunization with VP2/VP6 virus like particles, VP6 DNA and peptides induce protective immunity in various animal models (Chen et al., 1997; O'Neal et al., 1997; Ciarlet et al., 1998; Siadat-Pajouh et al., 2001). Both type specific and cross reactive T-cell epitopes have been mapped on the VP6 protein (Bruce et al., 1994; Franco et al., 1994). Moreover, passively delivered IgA monoclonal antibodies (MAbs) to VP6 have reduced chronic virus excretion in severe combined immunodeficient mice (SCID) (Burns et al., 1996).

**The core proteins**

Structural proteins VP1, VP2 and VP3 have affinity for ssRNA and function as core particles to hold the viral RNA. Each of the core proteins plays a role in the process of RNA transcription and replication. VP1 is a sub core protein present in few copies and has a calculated MW of 125 kDa. The protein product of rotavirus gene segment 1, it is the viral RNA polymerase and functions as both viral transcriptase and replicase (Stacy-Phipps et al., 1987; Mottion et al., 1994). However, VP1 requires VP2 for replicase activity (Zeng et al., 1996; Patton 1997). VP2, the major protein component of the core particles is encoded by rotavirus gene segment 2 and has a MW of 102 kDa. VP2 binds with the dsRNA and forms the replicase complex with VP1 and VP3 (Zeng et al., 1996). The replicase assays performed with mutant VP2 containing a deletion in the RNA binding domain suggest that during replication VP2 binds the mRNA template for minus strand synthesis (Patton et al., 1996). VP3 is a minor sub core protein encoded by rotavirus gene segment 3. It has a MW of 88
kDa and is found in the vertices of inner core. In addition to the RNA polymerase activity, VP3 possesses guanylyltransferase and methyltransferase activity and acts as multifunctional capping enzyme to cap viral and nonviral RNA in vitro (Chen et al., 1999).

The nonstructural proteins

Five segments of rotavirus genome code for six non structural proteins, NSP1 (gene segment 5), NSP2 (gene segment 8), NSP3 (gene segment 7), NSP4 (gene segment 10) and NSP5 and NSP6 (gene segment 11). All of these proteins (NSP1 to 3, 5, 6) except NSP4 are involved in replication. NSP1 is the most variable of all rotavirus protein and has been implicated in host range restriction in mouse (Broome et al., 1993). NSP2 has been reported to function as a molecular motor, catalyzing the packaging of mRNA into core (Taraporewala et al., 1999). NSP3 is a sequence specific RNA binding protein that binds the nonpolyadenylated 3' end of rotavirus mRNAs (Piron et al., 1999). It also interacts with the cellular translation initiation factor eIF4GI and competes with cellular poly (A) binding protein that leads to shutoff of cellular translation (Piron et al., 1999). NSP5 and NSP6 interact with each other and NSP2 during replication (Torres et al., 2000). NSP6 acts as a regulator in the self association of NSP5. NSP4 plays important role in viral morphogenesis and pathogenesis. It serves as intracellular receptor and permits DLPs to enter into ER for rotavirus maturation (Au et al., 1993). NSP4 is the first ever described viral enterotoxin that induces diarrhea in neonatal mice and rat via activation of intracellular Ca\(^{2+}\) mobilization (Ball et al., 1996; Morris et al., 1999).

2.5. Classification and strain diversity

Rotaviruses are antigenically complex and based on the antigenic specificity of the inner capsid protein VP6 they are classified into seven different groups (A-G). Of the seven groups, only groups A-C are known to infect humans, and group A rotaviruses cause severe life threatening disease in children worldwide. The group A rotaviruses (GAR) are further classified into SGI, SGII, SGI & II and neither SGI nor SGII on the basis of reactivity with SG specific MAbs directed to VP6 protein (Greenberg et al., 1983; Taniguchi et al., 1984). The two outer capsid proteins VP7 (glycoprotein) and VP4 (protease sensitive protein) elicit NAbs and mediate G and P serotype specificities respectively. Analogous to the classification of influenza viruses, rotavirus classification follows a binary
system that includes both G and P types. Neutralizing mouse monoclonal antibodies to VP7 protein have been extensively used for G typing in epidemiological surveys (Hoshino and Kapikian, 1996). On the other hand, neutralizing MAbs and hyperimmune serum samples that have been used to type VP4 had problems in sensitivity as well as cross reactivity. In recent years reverse transcriptase polymerase chain reaction (RT-PCR) genotyping has replaced the MAb based typing of rotavirus and epidemiological studies primarily involves molecular genotyping of rotaviruses (Iturriza-Gomara et al., 1999). All known G serotypes have been correlated with G genotypes; whereas, more P genotypes have been identified than P serotypes (Estes, 2001).

A total of 27 G and 35 P genotypes have been identified till date (Matthijnssens et al., 2011). The intensive epidemiological studies over the last four decades have shown that the incidence and distribution of group A rotavirus serotypes/genotypes vary between geographic areas during a rotavirus infection season, as well as from one such season to next. Surveys around the globe indicates that G1P[8], G2P[4], G3P[8], G4P[8] and G9P[6] or G9P[8] are the most common G and P type combinations causing disease in humans, while other G types (G8, G10, G12) and G/P combinations seem to be sporadic or only of regional relevance (Eichelberger et al., 2002; Santosh and Hoshino, 2005; Ray et al., 2007). The application of RT-PCR genotyping and sequencing has helped in identification of strain diversity, with >60 G-P combinations being recognized in human infections. In India, among the diarrhea hospitalizations, G1 and G2 are the commonest G types in combination with P[8] and P[6] or P[4] respectively (Kang et al., 2009). In addition, region specific neonatal infections with bovine human reassortants and unusual strains which may be evidence of zoonotic infection and/or reassortment have been reported (Ghosh et al., 2006; Ramani et al., 2007; Ray et al., 2007).

Accumulation of point mutations that leads to emergence of antibody escape mutants and genetic shift via exchange of genetic material through gene reassortment during dual infection of a single cell are the two prime mechanisms adding to the diversity and evolution of rotavirus (Iturriza-Gomara et al., 1999). Moreover, in a ecology where animal and human populations habitat in close association, zoonotic transmission and gene reassortment between human and animal rotaviruses also contributes to diversity of rotavirus. There has been continuous emergence of newer strains which cause more severe clinical disease
in infected neonates and children (Ramani et al., 2007). Thus, rotavirus diarrhea remains a major health problem both in developing and developed countries.

2.6. Replication

Rotavirus attachment and entry into enterocytes is a multifactorial process of interactions between the virus and cell surface and varies for different rotavirus strains (Mendez et al., 1999). Two most possible proposed mechanisms are, through (i) binding to receptors such as sialic acid and ganglioside GM1a via interaction with VP8 and integrins via interaction with VP4 and VP7 (Fukudome et al., 1989; Coulson et al., 1997; Guo et al., 1999; Hewish et al., 2000); (ii) Ca\(^{2+}\) dependent endocytosis or direct penetration of plasma membrane (Dowling et al., 2000). Rotavirus entry is accompanied by loss of VP4 and VP7, thereby internalizing transcriptionally active DLPs (Charpilienne et al., 1997). DLPs contain the RNA dependent RNA polymerase enzymes (VP1 and VP3 complex) which function as a transcriptase to synthesize the transcripts of full length positive strands (Mason et al., 1980; Sandino et al., 1986; Cohen, 1977). The plus strand RNAs that contain 5’ caps but lack 3’ poly (A) tails extrude from DLPs through the Channel I and are translated into 6 structural (VPs) and 6 non-structural proteins (NSPs) (Lawton et al., 1997). The plus strand RNAs also act as template for minus strand RNA leading to synthesis of dsRNA genome segments throughout the infection (Patton, 1986). RNA replication occurs concurrently with the packaging of the genome segment into newly formed cores. The viral proteins accumulate in an electron dense area of cytoplasm called viroplasm. The viral genome replication and assembly of progeny DLPs occur in the viroplasm. Subsequently, the DLPs via interaction with NSP4 bud directly into the lumen of endoplasmic reticulum (ER) (Au et al., 1993). In the ER, the DLPs acquire transient lipid membrane which is finally replaced by VP7 and VP4. NSP4, VP7 and VP4 proteins are synthesized in ribosomes in close relation to the ER and co-translationally inserted into the ER membranes (Estes, 2001). The rest of the structural and non-structural proteins are synthesized in free ribosomes of cytoplasm. The mature TLPs exit from the ER and the cells by two possible mechanisms involving (i) vesicle-associated vectorial transport system that leads to release of rotavirus particles from the apical pole of the cells before cell lysis (Jourdan et al., 1997) and (ii) cell lysis (Chasey and Lucas, 1977; Altenburg et al., 1980).
2.7. Epidemiology

Rotavirus infection occurs worldwide and causes 600,000 deaths in children annually (Glass et al., 2006). Although 82% of the total death due to rotavirus occurs in the developing countries, virtually every child experiences at least one episode of rotavirus gastroenteritis during the first few years of life (Parashar et al., 2006). Rotavirus infection is also responsible for 24 million clinic visits and 2.4 million hospitalizations, placing a significant economic burden on global health care system. Recent studies indicate that rotavirus causes approximately 39% of diarrhea associated hospitalization in children (Parashar et al., 2006). According to a recent report, India annually accounts for 122,000 to 153,000 deaths, 457,000 to 884,000 hospitalizations and 2 million out patient visits due to rotavirus infection in children under 5 years old and spends Rs.2 to 3.4 billion in treatment of rotavirus diarrhea (Tate et al., 2009). In fact, India accounts for 17% of the total worldwide rotavirus deaths (Jain et al., 2001b). The increased rate of rotavirus related mortality in the developing world is mostly due to inadequate health care systems and lack of management (Parashar et al., 2003). Although death due to rotavirus is few (20–60 in USA and 231 in Europe) in developed countries, morbidity among children is substantial resulting in hospitalizations (55000–70000 in USA and 87 000 in Europe) and out patient visit (200 000–272 000 in USA and 700 000 in Europe) (Parashar et al., 2006; Sariano-Gabarro et al., 2006). These statistics document that rotavirus disease alone constitutes a large proportion of child morbidity and mortality worldwide.

Genetic and antigenic diversity of rotavirus is high because of the segmented nature of the genome. Theoretically, from the known 27G and 35P types, >1378 different combinations of G and P proteins can be generated to create high serotype diversity. However, in practice, most combinations are not fit and do not survive subsequent rounds of replication in humans, so the actual number of G and P combinations is less than the possible number. Viruses circulating in humans now are characterized as being common human genotypes (G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8]), reассortants among human genotypes (G1P[4], G2P[8], and G4P[4]), reассortants between animal and human genotypes (G1P[9], G4P[6], G9P[8], and G12P[8]) and likely zoonotic introductions (G9P[6], G9P[11], G10P[11], and G12P[6]) (Gray et al., 2008). The common human genotypes represent the most prevalent viruses worldwide although their relative prevalence and distribution change with regard to location.
and time. In India, the strains of G2P[4] (25.7%) is the most common followed by G1P[8](22.1%), G9P[8](8.5%) and G12 (6.5%) with different P type combinations (Jain et al., 2001a; b; Kang et al., 2009). The very same investigators also reported frequent prevalence of unusual combinations such as G1P[4], G1P[6], G2P[6], G2P[8], G9P[4] and G9P[6] and mixed infections.

Rotaviruses are usually transmitted via the fecal/oral route; though transmission through respiratory droplets has also been suggested (Fragoso et al., 1986; LeBaron et al., 1990; Parashar et al., 1998). Transmission by the respiratory route does not, however, imply virus multiplication in the respiratory tract. Low dose, 10 to 100 infectious virus particles are needed to cause infection (Graham et al., 1987). This amount can readily be acquired from surroundings through contact with contaminated hands and objects. Resistance to physical inactivation along with large number of viral particles (1010 particles/gm) shed in feces facilitate efficient transmission of rotavirus (Breese et al., 2000). Additionally, susceptible children can acquire the infection from asymptomatic counterpart, adults and animals. Low birth weight and premature infants appear to be at greater risk for hospitalization due to rotavirus infection while malnutrition, with its probable associated immunodeficiency, is a predisposing factor for severe life threatening dehydration associated with rotavirus disease (Dagan et al., 1990; Newman et al., 1999).

In the temperate region of the world (mostly developed countries) rotavirus infection occurs primarily during the winter months of the year. On the other hand, in tropical countries rotavirus associated diarrhea is prevalent throughout the year with a peak in the colder months. Rotavirus winter epidemic is attributed to higher air borne transmission and indoor crowding along with maximum stability of the virus in low relative humidity (Cook et al., 1990). The peak incidence of rotavirus illness in children in tropical countries occurs between 6 and 11 months of age. In contrast, the highest rates of rotavirus diarrhea occur during the second year of life of the children in temperate countries (Bresee et al., 1999). This difference at the age of first attack has been proposed to be due to the seasonal nature of rotavirus infection in the temperate regions. In India, rotavirus infection in children is shown to be greatest (~40%) at the age of 6-23 months and lowest (13%) at <6 months of age (Jain et al., 2001b; Kang et al., 2009). Further, rotavirus positivity rates vary greatly in different settings - diarrhea hospitalization (20%), neonatal infections (35%), symptomatic and asymptomatic
infections in the community (15.1% and 6.3% respectively) (Ramani and Kang, 2007).

Nosocomial rotavirus infection represents a particular problem in neonatal nurseries around the world. Results from observational studies in industrialised countries indicate the rate of incidence of rotavirus gastroenteritis during hospitalization is 7.0 to 15.8/1000 child-days of hospitalization for children younger than 2 years, and 0.7 to 8.1/1000 child-days of hospitalization for children younger than 5 years (The pediatric rotavirus European committee 2006; Gleizes et al., 2006). In a systematic review of observational studies of rotavirus disease burden in Europe, it was estimated that for every 4 children <5 years of age admitted to hospital with community-acquired rotavirus gastroenteritis, there was 1 case of nosocomial infection (The pediatric rotavirus European committee 2006). Rotavirus accounts for 22.5% of nosocomial enteric infections in Indian setting (Ramani and Kang 2007).

2.8. Host Range
Rotaviruses are known to have wide range of host specificity infecting young one of many of mammalian (human, bovine, ovine, caprine, canine, equine, lapine, murine, simian) and avian (chicken, turkey, pheasant) species. There is significant restriction of growth of rotavirus from one species in another (host range restriction). However, evidences of natural interspecies transmission and reassortment in-vivo have been increasing (Palombo, 2002; Cook et al., 2004; Müller and Johne, 2007; Bányaï et al., 2009). The strains usually of animal origin such as G3 (in cats, dogs, monkeys, pigs, mice, rabbits and horses), G5 (pigs and horses), G6 and G8 (cattle), G9 (Pigs and lambs) and G10 (Cattle) have been isolated from human population throughout the world (Desselberger et al., 2001). Similarly, various human rotavirus G and P type combinations have also been reported in animal species. For example, G10P[11] has been detected in American and Canadian cattle population (Lucchelli et al., 1994) and in Indian cattle and buffalo (Gulati et al., 1999); G3P[6] and G4P[6] have been found in pig population in Poland and USA respectively (Winiarczyk et al., 2002).

2.9. Clinical symptoms and diagnosis
A sudden onset of symptoms is typically manifested in children infected with rotavirus after an incubation period of 1 to 2 days. The clinical presentation of rotavirus gastroenteritis includes watery, nonbloody diarrhea preceded by onset
of vomiting and subsequent presentation of fever and dehydration for 4-7 days (Uhnoo et al., 1986). Secondary infections with rotavirus are clinically milder or asymptomatic (Velazquez et al., 1996). Adults more frequently experience asymptomatic rotavirus infection. However, when symptoms are reported in adults, the most common are diarrhea, fever, headache, malaise, nausea or cramping (Anderson and Weber, 2004). Despite adults having milder symptoms of rotavirus infection, they are still infectious, and thus can act as reservoir and transmit the infection to susceptible children (Cox and Medley, 2003; Anderson and Weber, 2004).

Laboratory diagnosis of rotavirus infection primarily based on using enzyme immunoassay that involves detection of rotavirus VP6 group A specific antigen in diarrheal stools (Estes, 2001). Of the commercially available latex agglutination tests and ELISA kits for detection of rotavirus in stool, the latter are more commonly used system with 98 and 100% sensitivity and specificity respectively (Gilchrist et al., 1987). Although in the early rotavirus studies detection of RNA in polyacrylamide gel electrophoresis was an important for laboratory and epidemiological tool, this method is now used only to differentiate GAR from those of other groups. Rotavirus nucleic acid amplification by RT-PCR and real time PCR is the most sensitive technique currently used for the detection as well typing of rotaviruses recovered from stool specimens (Gouvea et al., 1990; Wilde et al., 1991).

2.10. Propagation in cell culture

Although fastidious in nature, various animal and human rotaviruses can be isolated and cultivated in cells of epithelial origin in vitro in presence of trypsin. Virus present in positive fecal sample can be isolated successfully in roller tube cultures with over 75% efficiency. Isolation of human rotaviruses was first achieved in fetal monkey kidney cell line, MA104 (Ward and Knowton, 1984). However, primary African green or cynomologus monkey cell culture and continuous human colon adenocarcinoma cell lines (CaCo-2 and HT-29) are reported to be more efficient than MA104 cultures for isolation and cultivation rotaviruses (Superti et al., 1991; Cumino et al., 1998).

2.11. Animal model

Neonates of several animal species (simian, bovine, porcine, ovine, canine, lapine and murine) have been used as experimental models to define
parameters of rotavirus infection, pathology, disease, immune response and test vaccines (Conner, et al., 1991; Conner and Raminng, 1996; Saif, et al., 1996; Saif, et al., 1997). Among these animals, particularly monkeys and pigs share a genetically and antigenically closer organo-physiology to human and have been described as the most appropriate models (Yuan and Saif 2002; McNeal et al., 2005). However, experiments with such animals may be prohibitively expensive on account of costly procurement and maintenance, usually requiring gnotobiotic conditions and cesarean delivery.

Mouse and rabbit are the frequently used animals to study rotavirus associated disease, pathology and immune response. The development of diarrhea have been reported to be age restricted occurring only in mice of <2 week old and rabbits of 1 week old age (Wolf et al. 1981; Raming 1988; Ciarlet et al. 1998). However, adult mice infected with selected strains of murine rotavirus have also been found to be susceptible to diarrhea (Burns et al. 1995).

Adult mice and rabbit models of rotavirus infection, without disease have been proved to be useful in assessing active immunity and protection after infection or vaccination with virus or virus like particles (Conner et al. 1993; Ciarlet et al. 1998).

2.12. Pathogenesis and pathophysiology of rotavirus infection

The pathogenesis and pathophysiology of rotavirus infections have been studied in various animal models. Rotavirus infects mature enterocytes in the mid and upper part of the villi in small intestine. Within 24 hrs of infection histological changes in absence of inflammation take place in the intestine depending on the animal species. In colostrum deprived calves, rotavirus infection leads to change the villus epithelium from columnar to cuboidal and the villi become stunted/blunted (Carpio et al., 1981). Along with villus blunting, thining of the intestinal wall has been reported in pig (Collins et al., 1989). In mouse, vacuolization of the enterocytes appear more pronounced with transient villus blunting (Starkey et al., 1986; Osborne et al., 1988). On the other hand, rabbits show no hiosogical changes in the intestine except marked imbalance in glucose and leucine uptake (Ciarlet et al., 1998). In contrast to animal studies, there are few studies that describe pathology of intestinal mucosa in infants with rotavirus infection. Studies of biopsy materials have revealed shortening and
atrophy of villi, distended ER, mononuclear cell infiltration, mitochondrial swelling and denudation of microvilli (Davidson and Barnes 1979).

It is likely that the fluid and electrolyte secretion caused by rotavirus infection is a multifaceted event attributed to several different mechanisms such as malabsorption secondary to enterocyte destruction, villus ischeemia, virus encoded toxin and stimulation of enteric nervous system. Rotavirus infects and destroys the mature absorptive enterocytes which are later on replaced by nonabsorptive crypt like secretory epithelium creating imbalance in net secretion (Moon 1994). Experimental evidences also support malabsorption of carbohydrate due to decrease expression of brush border disaccharidases such as lactase, sucrase, isomaltase etc. and the undigested sugars lead to induction of an osmotic diarrhea (Graham et al., 1984; Kapikian et al., 2001). Extensive vacuolation of the villi epithelium in mice has led a group of investigators to propose that fluid loss in rotavirus infection is secondary to local villi ischemia, at least in mice (Osborne et al. 1988). The NSP4 of rotavirus has been implicated to be a potent viral enterotoxin. Intraperitoneal injection of full length or cleavage product of NSP4 induces diarrhea in new borne mice (Ball et al., 1996; Estes and Morris 1999; Zhang et al., 2000). Electrophysiological studies suggested that NSP4 potentiates chloride ion secretion by triggering a calcium dependent signaling pathway (Angel et al., 1998; Morris et al., 1999). The clinical picture of rotavirus disease is not limited to diarrhea but involves nausea and vomiting which inspire the most recent hypothesis suggesting that rotavirus or its products induce intestinal fluid and electrolyte secretion by activation of enteric nervous system (Lundgren et al., 2000).

A recent advancement in pathology of rotavirus infection is that rotavirus can escape from the intestine into the circulation and replicates extra intestinally, with antigen and RNA detected in blood and peripheral tissues. Antigenemia and viremia have been identified as common event in acute phase of both natural and experimental infections (Blutt et al., 2003). In animal models rotaviruses have been shown to have ability to replicate in peripheral tissues: mesenteric lymph node (MLN), spleen, liver, lung and kidney to varying degrees (Crawford et al., 2006). Although there is no evidence that directly correlates with multiplication of rotavirus in extra intestinal tissues in natural infection, the discordance between genotypes detected in serum and stool from a single episode of rotavirus infection indirectly supports extra intestinal replication of the virus (Blutt et al.,
The clinical significance of the systemic spread of rotavirus is largely unknown while the association of rotavirus with central nervous system (CNS) disease symptoms has been confirmed in case reports (Iturriza-Gomara et al., 2002). Other diseases wherein rotavirus plays a possible role include type 1 diabetes and celiac disease (Honeyman et al., 1998; Honeyman et al., 2000; Stene et al, 2006).

2.13. Immunity against rotavirus

Despite decades long research, the exact mechanism or/and marker of immunity to rotavirus is poorly understood. Repeated infections seem to build resistance, however, immunity after natural rotavirus infection is incomplete and depends on time between two exposure, the properties of the strains involved and the immune status of the host at the time of each exposure (Bernstein et al., 1991). Studies with a variety of animal models have elucidated the relative importance of local and systemic immune responses in protection against rotavirus infection and disease. Rotavirus infection induces both innate and adaptive immune responses. Gastric acid and pepsin act as important defense factors against rotaviruses (Bass et al., 1992). Interleukin 1(IL1), alpha interferon (INFα) and gamma interferon (INFγ), cytokines induce a dose dependent resistance to rotavirus infection in-vitro, suggesting innate immunity may play role in modulating rotavirus infection (Bass et al., 1997). In addition, lactadherin a glycoprotein in maternal milk has also been assumed to be associated with passive innate mechanism to exert anti-rotavirus effect by inhibiting viral attachment to the receptors (Newburg et al., 1998).

Since primary site of rotavirus replication is enterocytes in vivo, the adaptive immune response is assumed to originate in and exhibit its effector function directly at the intestinal mucosa. The virus is processed by antigen presenting cells (APC) and presented to helper T lymphocytes (Th), cytotoxic T cells (CTLs) and B lymphocytes within the peyers patches and lamina propria of intestinal mucosa. Therefore, local mucosal immunity in the gut is believed to play a key role in protection. Antibodies are generally considered as good marker for infection and a proxy for protection in rotavirus infection. In a primary infection rotavirus specific IgM appears first in the luminal surface of intestine, followed by IgA and IgG (Grimwood et al., 1988). Intestinal antiviral IgA response is relatively shorter (1-4 weeks after infection) which is probably one of the factors that
contributes to multiple reinfection with rotavirus. During acute phase of infection, 
IgM serum antibodies predominate and are then replaced by IgG and, to a lesser 
extent, IgA. The serum rotavirus specific IgG response seems to be long lasting 
and when the titer is sufficiently high, it has been shown to mediate mucosal immunity by preventing virus cell attachment in the gut/ or blocking steps of the 
virus life cycle inside the infected enterocytes via transcytosis (Coulson et al., 
1990; Westerman et al., 2005). Following natural infection, titers of serum and 
intestinal rotavirus IgA as well as serum rotavirus IgG have been reported to 
correlate with protection (Matson et al, 1993; Giammarioli et al., 1996; Ruggeri et 
al., 1998; Jiang et al., 2002; Istrate et al., 2008).

Non antibody factors such as natural killer cells, CTLs, cytokines and 
other chemical mediators also confer protection/antiviral activity against rotavirus 
disease. CTLs have been shown to provide both active and passive immunity 
against rotavirus disease by helping in clearance of infected cells and thereby 
resolving infection in murine model. CD8+ T cells have been reported to mediate 
complete or partial protection from reinfections for a period of 8 months after 
primary infection (Franco et al., 1995). It has been demonstrated that adoptive 
transfer of splenic lymphocytes and CD8+ T cells from immunized animals to 
rotavirus infected suckling and SCID mice respectively could clear the infections 
in absence of rotavirus specific antibodies (Dharakul et al., 1990; Offit and Dudzik 
1990). Thus, both B and T cells play important roles in the immune response to 
rotavirus.

Antibodies against outer capsid proteins, VP4 and VP7 have been 
identified as crucial in imparting protection against rotavirus infection. It has been 
demonstrated that antibodies directed to VP4 prevent viral attachment to target 
cells thereby abrogating infection (Ruggeri and Greenberg 1991). On the other 
hand, antibodies against VP7 have been shown to prevent virion decapsidation 
and hence inhibit functional DLP formation (Ludert et al., 2002). Secretory IgA 
(SIgA) antibodies against inner capsid VP6, the most immunodominant protein in 
the viral capsid are very interesting as they have been shown to target more 
central aspects of rotavirus replicative cycle like inhibition of genome replication 
during transcytosis from the basolateral to apical surface of intestinal mucosa 
(Feng et al., 2002; Corthesy et al., 2006).

2.14.1. Oral Rehydration Therapy (ORT)

Current management of rotavirus diarrhea involves prevention and management of dehydration using oral or intravenous rehydration and promotion of breast-feeding for young infants. ORT coordinated by World Health Organization (WHO) has been able to significantly reduce the child mortality due to acute dehydrating rotavirus diarrhea (Victoria et al. 2000). Recently, the WHO and UNICEF has also recommended routine use of zinc for 10-14 days in the management of diarrhea in children, irrespective of etiology (Fontaine, 2006).

2.14.2. Antidiarrheal agents

Several organic (Kureha, a protein bound polysaccharide; Ovocystatine, a cysteine protease inhibitor; Aprtinin, serine protease inhibitor) and chemically synthesized compounds (Ribavirin; 9-S-2, 3-Dihydroxylopropyl adenine; NMSO₃, a sulfated sialyl lipid) and plant extracts (Theaflavins extracted from tea; Enkaphalinase inhibitors from reccadortil) have been found to be effective for suppression of rotavirus infection in vitro and in vivo (Ebina et al., 1990; Clark et al., 1998; Salazar-Lindo et al., 2000; Estes 2001; Takahashi et al., 2002). Some of them have prevented HRV induced diarrhea in suckling mice and humans, but none of them has yet been in clinical use.

2.14.3 Probiotics

Probiotics are “live organisms that when administered in adequate amounts confer a health benefit on the host (FAO/WHO 2002).” In the last few decades, the use of probiotics has gained considerable attention as a safe and accessible adjunctive therapy in treatment of for gastrointestinal (GI) diseases (Isolauri et al., 1994; Bibiloni et al., 2005). Several microorganisms that include *Lactobacillus rhamnosus*, *L. plantarum*, several strains of bifidobacteria, *Enterococcus faecium* SF68, the yeast *Saccharomyces boulardii* and preparations containing a mix of strains are effective in reducing the severity and duration of diarrhea in children (Allen et al., 2004). A recent study in Italy has reported that probiotics are the most commonly prescribed treatment in children with diarrhea (Fontana et al., 2004). The therapeutic effect of certain probiotic bacteria (*L casei* strain GG, microbacteria, *Lactobacillus reuteri*) against rotavirus gastroenteritis has been suggested to be due to their ability to stabilize and reinforce the mucosal brush border (Schiffrin et al., 2002), release antimicrobial
substances (Ganzle et al., 2000) and stimulate the local antigen specific and non specific immune responses (Kaila et al., 1995; Schiffrin et al., 2002).

2.14.4. Active immunization - rotavirus vaccines

Much alike the other pathogens of economic and public health importance, vaccine is being identified as the best current strategy to decrease the burden associated with severe and fatal rotavirus diarrhea. Many groups concerned with human health including WHO, Institute of medicine and Global Alliance for Vaccines and Immunization (GAVI), Program for Appropriate Technology in Health (PATH) have emphasized inclusion of rotavirus vaccine in childhood immunization program. Two new generation rotavirus vaccines, Rota Teq and Rotarix are launched in the global market and licensed over 100 countries. These vaccines have been shown to be safe and effective to reduce the rate of severe rotavirus disease (Dennehy 2008). The new generation rotavirus vaccines are primarily of attenuated human animal rotavirus reassortants and common and uncommon human rotavirus isolates.

Rota Teq, manufactured by Merck Inc. USA is a pentavalent vaccine containing five bovine human reassortants developed from WC3 bovine strain (G6P[5]), representing the common human VP7 types G1-G4 and most common VP4 type, P[8]. Efficacy trials showed 74% and 98% protection against all and severe disease respectively without increased risk of intussusceptions among the vaccinees compared with placebo recipient (Vesikari et al., 2006a; Block et al., 2007). This vaccine has been approved by the Immunization Practices Advisory committee (ACIP) for inclusion in routine immunization of infants in USA and other countries. Another tetravalent bovine (strain UK, G6P[7]) human rotavirus reassortant vaccine (by Wyeth Ayerst, USA) representing common human VP7 serotypes have been developed and trials in US have demonstrated satisfactory level of attenuation, safety, infectivity and immunogenicity (Vesikari et al., 2006b). The vaccine has been found to provide 90% protection against severe rotavirus disease. Its development has been taken over by manufacturers in Brazil, India and China. Rotarix, a monovalent human rotavirus strain 89-12 (G1P1A [8]) derived vaccine is developed by Glaxo Smith Kline Biologicals, Belgium and first to be trailed in multiple settings. The efficacy has been estimated to be 86-96% against severe rotavirus disease (Vesikari et al., 2007). There was no record of
significant adverse events or increased risk of intussusceptions among the vaccinees.

Development of candidate rotavirus vaccines from certain naturally attenuated human neonatal rotavirus strains is being under way. RV3, a G3P2A[6] strain isolated from asymptomatic neonate in Melbourne, Australia has been developed into a vaccine and demonstrated to induce immune response in 46% of the vaccinees protecting from rotavirus disease in early Phase I and Phase II trials. Currently, Biopharma, Bandung, Indonesia is working on this vaccine. Two more natural bovine human reassortant strains 116E (G9P[10]) and I321 (G10P[11]) isolated from symptomatic neonatal rotavirus infections from India are being under development as candidate vaccines. These two neonatal Indian vaccines have interesting differences in their genomic structure. Strain 116E is a human strain with a single bovine VP4 while I321 is composed primarily of bovine genes and has only two segments of non structural genes of human origin (Bhan et al., 1993; Dunn et al., 1993). In a small placebo-controlled trial of both vaccines, only 116E was found to elicit significant immune response and therefore, the strain I321 was shelved and only 116E is being pursued actively. The vaccine is under trial in India by Bharat Biotech/Indo-US consortium.

In addition to the development of orally administered live attenuated vaccines, other approaches such as different types rotavirus antigen (virus like particles, cold adapted strains, inactivated strains and DNA vaccines) and routes of administration (intranasal, intrarectal, intramuscular) are being evaluated and tested in animal models (O'Neal et al., 1997, 1998; McNeal et al., 1998, 1999; To et al., 1998; Yuan and Saif 2002; Angello et. al., 2006, VanCott et al., 2006). Although, the new generation rotavirus vaccines are undoubtedly useful to ensure an improved and healthy early childhood, introduction of these vaccines in regular childhood immunization schedule of developing countries confronts certain practical challenges, which for there is always a need of alternative/adjunct means for the management of rotavirus diarrhea.

2.14.5 Passive immunization

Utilization of immune serum from immunized animals or convalescent humans for prevention and treatment of infectious disease (serum therapy) (Buchwald and Pirofski, 2003) is the oldest antimicrobial therapy. However, passive immunization by oral administration of specific antibodies is a recent and
attractive concept in human medicine to establish passive protection/immunity against GI pathogens. During the last two decades oral delivery of specific antibodies prepared against variety of enteric pathogens has been tested with various degree of success in both animal and human (Pathak and Haque 2003).

The passive transfer of maternal secretory IgA (SIgA) through breast milk has important implications in neonatal period for protection against GI infections including that of rotavirus. The protective efficacy of breast milk correlates positively with the concentration anti-rotaviral SIgA (Asensi et al., 2006). Breast fed infants tend to excrete less rotavirus than bottle fed infants do after infection with rotavirus (Duffy et al. 1986). Moreover, virus specific antibodies, IgA and IgG in the lumen of the small intestine have been regarded as the primary determinant of resistance to rotavirus disease (Ruggeri et al., 1998; Westerman et al., 2005). Hence, transfer of passive immunity through orally delivered immunoglobulins is a viable prophylactic strategy.

2.14.5.1 Human serum immunoglobulins

Oral supplementation of pooled plasma derived immunoglobulin (IgG) preparations was shown to have beneficial prophylactic and therapeutic effects in terms of reduction of duration and severity of rotavirus diarrhea in children (Barnes et al., 1982; Guarino et al., 1994). The use of human gammaglobulins, however, is limited due to the risk of viral contamination and high production and storage cost.

2.14.5.2 Bovine colostral antibodies

Bovine antibodies are actively transported from plasma to milk in cows and present in high concentration in colostrums. These antibodies protect calves from GI infections during the neonatal period and are of major importance for their survival. A cow produces about 1.5 kg antibodies in the first few days after calving, making it an attractive source of large scale production of IgG. Bovine antibodies consist of three classes of IgG subclasses – IgG1, IgG2 and IgG3 along with IgM, IgD, IgA and IgE (Butler et al., 1983; Rabbani et al., 1997). In contrast to human breast milk in which IgA is the dominant immunoglobulin, IgG1 is the dominant immunoglobulin in bovine colostrum (Butler et al., 1998). Though the titers of specific antibodies against human pathogen is low in colostrum from unimmunized cows, it is possible to produce high titers of specific antibodies in hyperimmunized bovine colostrum (HBC) following vaccination of pregnant cows.
with antigen of choice including rotavirus (Brussow et al., 1987; Hilpert et al., 1987). HBC has been effectively used for prophylactic and therapeutic benefits in rotavirus diarrhea with reduced viral excretion, stool out put and need for rehydration in hospitalized children (Mitra et al. 1995; Davidson et al. 1989). Gastrogard-R, a HBC preparation containing anti-rotavirus immunoglobulins launched by Anadis Lab (Melbourne, Australia and Washington) has been recommended by WHO to complement global rotavirus vaccine initiatives for millions of children without access to rotavirus vaccine, such as children who are too young or old to receive vaccine priority as well as children at risk from epidemic disease in refugee camp, hospitals or day care centers (http://www.medicalnewstoday.com/articles/100267.php). However, up till now its availability is restricted in developed countries because of high cost of production.

2.14.5.3 Llamma antibodies

In 1990s biologists in Belgium discovered that unlike other mammals, the camalid family which includes llamas, camels and alpacas produce two types of antibodies: the conventional Y shaped IgG molecules consisting of four protein chains and smaller two chain antibodies. The second unusual type of antibodies that are devoid of light chains are referred as “heavy chain” antibodies. In contrast to IgG which consists of an antigen binding variable domain of the heavy chain (VH) and the light chain (VL), the antigen binding domain of heavy chain antibodies consists of only the variable domain of heavy chains (VHH). These antibodies have been shown to possess great potential in biotechnological applications because of their unique characteristics involving production, folding, stability and affinity (van der Linden et al., 1999). Recently, these antibodies have been shown to protect infant mice from rotavirus induced diarrhea when used as both prophylaxis and therapy (Pant et al., 2006). The technology awaits the decision to use VHH antibodies against infectious diseases in human.

2.14.5.4 Chicken egg yolk antibodies

In 1893, Klemperer discovered that immunized hens transfer “neutralizing proteins” (later on termed as IgY) to the egg yolk. However, egg antibody research was not a main focus until issues of animal welfare become a topic of concern due to the publication of Russel and Burch’s The Principle of Humane Experimental Technique in 1959, which discusses the pain and distressful methodologies involved in animal experimentation including collection of large
volume of blood for antibody separation and suggests for replacement and refinement in the techniques. IgY is over a century old technology that opened up new area of egg antibody research. The technology steadily improved through 1980's and has yielded enhanced conditions, methodologies and insight into the seemingly endless application of egg antibodies in the areas of diagnostics, immunoprophylaxis and therapy against infectious diseases, antibiotic alternative therapy, xenotransplantation etc.

IgY antibodies are the predominant serum immunoglobulin in birds, reptiles and amphibian, and are transferred from serum to egg yolk in the female to confer passive immunity to embryos and neonates, a process very much alike to placental IgG transfer in mammals which confers passive immunity to fetus and new borne (Patterson et al., 1962; Klemperer 1893). IgY is the functional equivalent of mammalian IgG. The nomenclature of “IgY” was initially proposed by Leslie and Clem due to its enrichment in egg yolk (Leslie and Clem, 1969). From the evolutionary perspective, IgY antibodies are considered to be the ancestor of mammalian IgG and IgE antibodies (Warr et al., 1995).

2.14.5.4.1 Structural characteristics

Although the IgY antibodies are functional homologue of mammalian IgG, there are some profound differences in their chemical structures. IgY consists of two heavy (H) and two light (L) chains with a molecular mass of 180 kDa, larger than that of mammalian IgG (150 kDa) (Shimizu et al., 1993; Sun et al., 1993). The greater mass of IgY is due to an increased number of heavy chain constant domains and an extra pair of carbohydrate chains. The heavy chain of IgY present 5 domains – the variable domain (VH) and four constant domains (Cu1, Cu2, Cu3 and Cu4), unlike mammalian IgG which has three constant domains (Cy1, Cy2, and Cy3) (Figure 2.2). The amino acid sequence comparison between IgY and IgG have revealed that the Cy2 and Cy3 domains of IgG are closely related to the Cu3 and Cu4 domains of IgY. On the other hand Cu2 domain is absent in γ chain which is considered to form the hinge region of IgG (Warr et al., 1995). The hinge region of IgY is smaller and less flexible compared to that of IgG. In addition, IgY is a more hydrophobic molecule than IgG (Davalos-Pantoja et al., 2000). The structural and amino acid sequence differences determine the biochemical features and immunological functions of the two types of antibodies.
2.14.5.4.2 Biochemical properties

Egg antibodies have unique biochemical properties and few such properties are described below.

(i) High affinity and avidity of IgY antibodies

Chicken IgY antibodies against bacterial and human proteins are found to have high affinity (the strength of the reaction/sum of the attractive and repulsive forces operating between a determinant on the antigen and a combining site on the antibody) and avidity (the measure of overall strength of binding of an antigen with many antigenic determinants and multivalent antibodies or sum of the individual affinities) (Ikemori et al., 1993; Lemamy et al., 1999). This is thought to be due to evolutionary divergence between the immunogen and immunizing host, or due to different affinity maturation process of IgY (Bezzubova and Buerstedde 1994; Warr et al., 1995).

(ii) Non reactivity to mammalian complement factors and Fc receptors

Egg yolk immunoglobulins neither activate mammalian complement factors nor interact with mammalian Fc receptors that could mediate inflammatory response in the GI tract (Barkas and Watson 1979; Carlander et al., 1999).

(iii) Resistance to extreme pH and temperature

Usually IgY is stable at pH 4-9 and temperature 65°C in aqueous condition (Hatta et al., 1993). However, the resistance of IgY to extreme pH ranges (pH 2-3 in acidic and 11-12 in alkaline conditions) increases if high salt conditions or stabilizing agents such as sorbitol are present (Lee et al., 2002). IgY has been reported to be stable at 4°C for more than a year (Jensenius et al., 1981).
Advantages of using egg as source of antibody

Chicken as a host for immunization and their eggs as a source of antibodies carry several attractive advantages.

(i) Better and persistent immune responsiveness

Birds produce enhanced level of antibodies against proteins of phylogenetically distant donor organisms with much lesser quantity of antigen as compared to other mammals require to produce an efficient immune response (Gassmann et al., 1990). The avian immune response has been also shown to be persistent with high and long lasting titer of specific IgY antibodies in the egg yolk of immunized hens (Gassmann et al., 1990; Hatta et al., 1993).

(ii) Non invasive collection of antibodies

Collecting eggs from immunized hens for isolation of antibodies is a non-invasive, non-stressful process. This is not only a much easier and more reliable procedure, but also a method of biological production favorable to animal welfare (Van Regenmortel 1993).

(iii) Simple and economical isolation process

The isolation of IgY antibodies involves separation of yolks from egg whites, followed by the purification of antibodies in yolk from lipids and other materials. The materials and methods that have been developed include polyethylene glycol (PEG) precipitation, DEAE fractionation, chloroform extraction, water dilution, precipitation with dextran sulphate or Dextran blue or xanthan gums, separation in a two phase system (phosphate and Triton-X-100), a freeze thaw cycle coupled with gel filtration on Biogel P-150 (Polson et al., 1980; Akita nad Nakai 1993; Schwarzkopf and Thiele, 1996; Bizhanov and Vyshniauskis,2000; Stalberg and Larsson, 2001; Devi et al, 2002). The isolation procedures are generally efficient and economical with varying yields, purity, stability and activity of IgY.

(iv) Large yield and scalable production

A hen usually lays about 280 eggs in a year and egg yolk contains 100-150 mg of IgY per yolk suggesting that more than 40 gm of IgY per year can be obtained from each chicken through eggs. It has been shown that antigen specific IgY antibodies constitutes 2-10% of the total IgY harvested (Schade et al., 1994). Since the housing and caring for millions of chicken is well developed in poultry industry, the large scale production of IgY antibodies can avail economic viability.
2.14.5.4.4 Prophylactic and therapeutic uses of egg yolk antibodies against infectious diseases

Keeping in view of the inherited advantages of IgY, currently, chicken eggs have gained considerable attention as a convenient source of specific antibodies for prevention and treatment of a number of GI infections. The best known examples include the treatment of diarrhea caused by enterotoxigenic Escherichia coli and Salmonella species in animals. The E. coli induced diarrhea in three day old piglets has been cured after treatment with egg yolk antibodies obtained from hens immunized with antigens from enterotoxigenic E. coli while those treated with egg yolk powder from unimmunized hens continued to have diarrhea with 62.5% deaths (Marquardt et al., 1999). In field trial as well, the incidences and severity of diarrhea in 14-18 day old weaned piglets fed with egg yolk antibodies have been found to be much lower than in those fed with a commercial diet containing antibiotic (Marquardt et al., 1999). An earlier in-vitro study by Jin et al. (1998) has demonstrated that purified antibodies against E. coli from the yolk of chicken were able to block the binding of E. coli to mucosal receptor. It has been shown that unfractionated hen egg yolk can significantly reduce the attachment of Salmonella Typhimurium to murine intestinal epithelial cells in vitro (Deignan et al. 2001). Oral administration of whole egg powder containing S. Enteritidis specific antibodies could reduce the rate of S. Enteritidis contamination of eggs laid by experimentally infected hens (Gurtler et al. 2004). In multi-trial study, Kassaify et al (2004) have shown that feed supplementation with egg powder from immunized hens resulted in the rapid decrease in fecal excretion of S. Enteritidis and elimination of the organism after 2 weeks of feeding. Recently Rahimi et al. (2007) has described that the birds treated with S. Enteritidis specific IgY showed significantly lower fecal shedding of S. Enteritidis.

Other economically important poultry and animal pathogens against which specific IgY antibodies have shown positive results are Marek’s disease virus (MDV), infectious bursal disease virus and Mycoplasma gallisepticum (infectious coryza), Campylobacter jejuni and Pythium indiosum (Kermani Arab et al., 1976; Tsubokura et al. 1997; Rangel et al., 2010; Yousif et al., 2006; Elsheikha et al., 2008). Currently, attempts are being made to use egg antibodies as feed additives to replace the use of antibiotics in animal feedstuff, a regular practice in veterinary medicine. Protective efficacy of IgY antibodies have been tested against fish diseases too. Specific immunoglobulins from hens have shown 60%
protection in Carassius auratus Gibelio against challenged with Aeromonas hydrophila (Li et al., 2006). IgY has been also explored for the use of antivenoms and toxoids (Prabhu et al., 2010; Venky’s India Ltd, personal communication) in order to substitute large animals such as horses as a source of antibodies.

The passive protection with IgY has been reported against a number of diseases of public health importance. The IgY-Hp obtained from hens immunized with Helicobacter pylori could provide a novel alternative approach to treat antibiotic resistant H. pylori infection (Ji Hyun et al., 2002). Streptococcus mutans, the causal agent of dental plaque could be inhibited by using mouth wash containing specific IgY antibodies (Tokoro 1992; Hatta et al., 1997). It has also been shown that gargling with a yolk preparation specific to Pseudomonas aeruginosa once a day prevents the colonization of these bacteria in the lungs of patients with cystic fibrosis (Carlander et al. 2000). Chronic colonization with Pseudomonas aeruginosa in the airways is the principal cause of high morbidity and mortality of patients with cystic fibrosis. In the first trial of oral antibody, no positive cultures were found when the patients received the treatment, suggesting that treatment with antibodies from eggs against Pseudomonas aeruginosa has a prophylactic and therapeutic effect. The therapeutic property of IgY has also been used for the treatment of vascular disorders (arteriosclerosis and atherosclerosis) and cancer (Yang et al 1997; Stolle and Beck 1999). More recently IgY antibodies have also been shown to be effective against H5N1 and H1N1 strains of influenza virus (Nguyen et al., 2010). Even visceral candidiasis has been reported to be inhibited by Anti-CAIgY antibodies in immunodeficient mice (Ibrahim et al., 2008).

Oral administration of IgY antibodies have also been proven effective for passive protection against enteric viral diseases. In studies of coronavirus induced diarrhea in calves, yolk immunoglobulins have been reported to be four times more efficient than those from colostrums in lowering intestinal viral titers and inhibiting diarrhea and mortality (Ikemori et al., 1997). Anti-rotavirus IgY antibodies were reported to be beneficial against rotavirus induced diarrhea in mice (Ebina et al.1996, Sarkar et al., 2007) and calves (Kuroki et al., 1993; Vega et al., 2011). A single study on the use of IgY antibodies in the treatment of rotavirus diarrhea in hospitalized children has reported modest improvement characterized by an earlier clearance of rotavirus from the stools (Sarkar et al., 2001). However, the production of specific IgYs against common HRV serotypes,
G1-G4 and G9, and their evaluation detailing the effect on the intestinal viral load and cellular pathological changes have rarely been described in the previous studies.