Rotavirus diarrhoea in Buffaloes: epidemiology, pathogenesis and prophylaxis

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ABSTRACT - Globally, rotavirus infection is the most important cause of severe diarrhea in infants and animals. In this report, we review the results of pathogenesis studies, strain surveillance and characterization studies published and discuss new insights gained from these studies on the potential mechanisms of the evolution and spread of new rotavirus strains. Early epidemiological studies in Italian buffalo herds revealed the predominance of strains with G8 specificity and detected strains with the rare, RRV-like, VP4 P[3] genotype. In an our previous study 125 fecal samples were collected from buffalo calves affected with diarrhoea, in seven dairy farms in Southern Italy. Rotaviruses were detected in 21 samples (16.8%) by an immunochromatographic assay and by reverse transcription-PCR (RT-PCR). Analysis of the VP7 gene revealed that 57% (12 of 21) of the isolates were G6, 23.8% were G8 (5 of 21) and 19% (4 of 21) were G10. Analysis of the VP4 revealed that 71.4% (15 of 21) of the isolates were P[5] and that 28.6% (6 of 21) were P[1]. The most common combination of G and P types was P[5],G6 (57%), followed by P[1],G10 (19%), P[5],G8 (14%) and P[1],G8 (9.5%). While P[5],G6 rotaviruses are very common in Italian bovine herds, the antigenic combination P[1],G10 is unusual and presumably derives from reassortment between P[1] and G10 strains, that appear to be more frequent in buffaloes and bovines, respectively. The presence of bovine-like G and P serotypes suggests that in Italy the epidemiology of buffalo rotaviruses overlaps the epidemiology of bovine rotaviruses, presumably because of the strict species affinity and/or of the intermingled distribution over the same geographical areas of the buffalo and bovine herds.

Key words: Rotavirus, Bubalus bubalis

INTRODUCTION - Acute diarrheal diseases are still a major health problem throughout the world, causing 25–30% of all deaths among children younger than 5 years of age in developing countries (Estes MK., 2001) while in developed countries are associated with considerable morbidity and a substantial number of hospitalizations among children and the elderly. Diarrheal diseases are also of Veterinart public health importance. Acute diarrhoea can be caused by many different agents including parasites, bacteria and viruses; the latter of which have been given significant attention in recent years. This review is devoted to Rotavirus-induced enteric infection, and in particular, to the pathophysiological mechanisms proposed to underlie the intestinal fluid secretion caused by the virus and strain surveillance and characterization studies published in water buffalo.
**ROTA VIRUS STRUCTURE AND SEROTYPES** - Rotaviruses, which comprise a genus of the Reoviridae family, have a capsid with 3 protein layers that encase a genome with 11 segments of double-stranded RNA (dsRNA) (Estes MK., 2001). Each segment usually codes for a single structural or nonstructural protein. The inner layer encasing dsRNA is composed of the VP2 protein and small numbers of the VP1 and VP3 proteins, which are associated with the genomic RNA. This core is surrounded by the middle protein layer, which is composed entirely of VP6, the antigen that defines group and subgroup (SG) specificities. The outer capsid layer consists of the VP7 glycoprotein layer, in which VP4 spikes are embedded. The 2 outer capsid proteins carry rotavirus serotype (neutralization) specific antigens and are encoded by segments 4 (VP4 protease-sensitive protein and P serotype antigen) and by segments 7, 8, or 9 (VP7 glycoprotein and G serotype antigen).

Because the VP4 and VP7 proteins are encoded by separate gene segments, rotaviruses can generate new P-G serotype antigen combinations through reassortment after dual infection of single cells. Because both serotype antigens are believed to be key in the development of protective immunity, it is necessary to assess their prevalence and to study genetic and antigenic variation for both G and P serotypes. Although there is good evidence from animal experiments that passively transferred VP7- and VP4-specific antibodies protect separately, it is less clear which component of the immune response is most important for protection after natural infection or vaccination (Offit et al., 1986).

At least 15 G serotypes and 25 P serotypes and subtypes have been identified. Because identification of P serotypes is technically difficult, usually, only the corresponding VP4 genes are identified by genotyping methods in surveillance studies. To distinguish strains that have been identified by genotyping only from those identified by P-serotyping, a dual nomenclature is used. P genotypes are expressed as “P,” followed by a number in brackets (e.g., P[6]), whereas P serotypes are designated by “P” with a serotype number, followed by the corresponding genotype in brackets (e.g., P2A[6]) (Estes MK., 2001).

Rotaviruses can also be classified according to their VP6 SG specificity (I or II), by use of an EIA with monoclonal antibodies (MAbs) or by nucleotide sequencing of a VP6 gene fragment (Greenberg et al., 1983; Iturriza-Gomara et al., 2002), and according to their RNA profile (long or short electropherotype), by use of acrylamide gels and on the basis of the migration rate of gene 11. The short-electropherotype phenotype results from a partial duplication in gene 11, which causes it to migrate more slowly than gene segment 10, whereas the standard-sized gene 11 of long-electropherotype strains migrates faster than segment 10 (Matsui et al., 1990).

The serotypes of rotavirus needed to be reconsidered when VP4, the other outer capsid protein, was identified as an independent serotype antigen (P serotype) by sequencing of VP4 genes from strains with different G serotypes and by neutralization studies (Hoshino et al., 1985; Gorziglia et al., 1988, 1990). P serotypes were also shown to be important for protective immunity by studies of animal models in which reassortants containing HRV P serotypes and animal rotavirus G serotypes were used (Offit et al., 1986). However, serotyping was a challenge in the absence of a collection of MAbs specific for the diversity of P serotypes. Although MAbs to several common P serotypes were identified, cross-reactivity between the types precluded their use for routine P serotyping (Coulson BS., 1993; Padilla-Noriega et al., 1993).

New methods that greatly facilitated strain surveillance studies, including reverse-transcription polymerase chain reaction (RT-PCR) genotyping and automated nucleotide sequencing, have been widely used for this purpose since the early 1990s. A multiplexed,
seminested RT-PCR method to identify G serotypes by genotyping (Gouvea et al., 1990) permitted the detection of both the common serotypes (G1G4) and the rare serotypes (e.g., G8 and G9) for which EIA-based serotyping antibodies were either not available or not in routine use. Finally, these multiplexed RT-PCR methods were extended to permit genotyping of the other major neutralization protein, the P protein (VP4) (Gunaseena et al., 1993; Gentsch et al., 1992). As for G-genotyping, common P types (P[4] and P[8]) and newly identified rare P types (P[6], P[9], and P[10]) could be detected. Analogous hybridization methods for genotyping were also developed (Flores et al., 1990, Larralde et al., 1990).

**TRANSMISSION** - Rotaviruses are transmitted by the fecal-oral route. Only 10 to 100 infectious virus particles are needed to cause infection. This amount can readily be acquired through contact with contaminated materials and objects. Large numbers of viruses are shed in fecal matter, from 100 to 1000 particles per milliliter, so contamination of objects is relatively easy. Additionally, virus excretion occurs in asymptomatic individuals. Notably, standard sanitary measures that kill most bacteria and parasites are ineffective in controlling rotavirus, as demonstrated by the fact that rotavirus incidence is similar in countries with both high and low sanitation standards (Estes MK., 2001).

**PATHOGENESIS: THE MOLECULAR BASIS OF DIARRHEA INDUCTION** - Rotavirus diarrhea is multifactoral, has malabsorption and secretion components, and may have other components suggested to be related to villus ischemia and intestinal motility. Here relevant data on the induction of each of these components are presented.

**Malabsorption**. A malabsorptive component of rotavirus diarrhea appears to be related to the primary infection with the virus. Infection of villus enterocytes leads to a cascade of events involving Ca\(^{2+}\). This disruption of Ca\(^{2+}\) homeostasis appears to be mediated by synthesis of viral proteins (del Catillo et al., 1991; Michelangeli et al., 1991). Increased Ca\(^{2+}\) permeability at both the plasma membrane and the endoplasmic reticulum leads to an increase in \([\text{Ca}\(^{2+}\)]\(_i\)\), triggering a chain of events that leads to cell lysis (Perz et al., 1998). The fact that NSP4 expressed in cells also leads to increases in \([\text{Ca}\(^{2+}\)]\(_i\)\) implicates it as the mediator of virus-induced \([\text{Ca}\(^{2+}\)]\(_i\)\) dysregulation (Tian et al., 1994). A fragment of NSP4 (amino acids 112 to 175) is secreted via a nonclassical pathway early after infection (Zhang et al., 1999), and this fragment added exogenously to cells also causes an increase in \([\text{Ca}\(^{2+}\)]\(_i\)\) (Tian et al., 1995). The increase in \([\text{Ca}\(^{2+}\)]\(_i\)\) follows NSP4 binding to a specific apical receptor (Estes MK., 2003) that triggers a PLC-IP\(_3\) cascade resulting in release of Ca\(^{2+}\) from intracellular stores (Dong et al., 1997). In contrast, the increase in \([\text{Ca}\(^{2+}\)]\(_i\)\) induced by intracellular NSP4 is independent of PLC stimulation (Tian et al., 1995). The NSP4-mediated effects may amplify the diarrheagenic effect of infection in the absence of significant visible tissue damage. However, the ability of inactivated rotavirus particles to induce diarrhea (Shaw et al., 1995) suggests that viral structural proteins may also play a role in the dysregulation leading to diarrhea.

Rotavirus infection has other effects on enterocytes that may contribute to malabsorption. Infection leads to an increase in \([\text{Na}\(^+\)]\(_i\)\) and a decrease in \([\text{K}\(^+\)]\(_i\)\), which appear to be related to increased plasma membrane permeability and not inhibition of the Na\(^+\)/K\(^+\) pump (del Castillo et al., 1991). Changes in intracellular levels of Na\(^+\) and K\(^+\) could impair electro-neutral NaCl absorption and Na\(^+\)-linked nutrient absorption, resulting in a loss of fluid.
[Na⁺]i dysregulation may be related to a general inhibition of the Na⁺-solute cotransport systems (Halaihel et al., 2000a). NSP4 may also be involved, as the NSP4₁₁₄₋₁₃₅ peptide is a specific and noncompetitive inhibitor of SGLT1 (Halaihel et al., 2000b). Infection also reduces the expression of digestive enzymes at the apical surface of infected enterocytes. For example, the activities of alkaline phosphatase, lactase, sucrase, and maltase are reduced (Brunet et al., 2000, Collins et al., 1988; Davidson et al., 1977). The expression of sucrase and isomaltase in cultured human intestinal epithelium was also reduced, probably as a result of perturbation of protein targeting and the microvillar cytoskeleton (Jourdan et al., 1998). Rotavirus infection alters the structure of polarized enterocytes in a number of ways. The increase in [Ca²⁺]i induced by rotavirus infection affects the Ca²⁺-sensitive proteins F-actin, villin, and tubulin, damaging the microvillar cytoskeleton, whereas rearrangements of other cytoskeletal proteins (cytokeratin-18) are independent of changes in [Ca²⁺]i (Brunet et al., 2000 a, b). Both rotavirus infection and NSP4 promote functional changes in tight junctions between enterocytes that maintain the epithelial barrier (Dickman et al., 2000, Tafazoli et al., 2001). The drop in transepithelial resistance induced by either the virus or NSP4 suggests that infection can cause paracellular leakage. Rotavirus also induces intestinal epithelial cells to secrete CXC and CC chemokines, suggesting that enterocyte chemokine secretion plays a role in initiating the immune response to infection (Casola et al., 1998; Rollo et al., 1999; Sheth et al., 1996). Interleukin-8 (IL-8), GRO-, RANTES, interferon (IFN)-stimulated protein 10, and granulocyte-macrophage colony-stimulating factor (GM-CSF) are stimulated, whereas other chemokines (tumor necrosis factor alpha, IL-1, IFN-, IFN-, MIP-, MCP-1, and IL-6) are unchanged. Induction of IL-8 and RANTES is noteworthy because these are the most potent chemoattractants for intestinal intraepithelial lymphocytes (Ebert E., 1995). It is unclear if virus replication is required for induction of chemokine secretion (Casola et al., 1978; Rollo et al.,1999). In addition, the levels of PGE₂ are increased in infected intestine (Zijlstra et al., 1999). The chemokines may activate the immune response rather than directly contribute to diarrhea. Thus, rotavirus infection causes a number of changes in the villus epithelium that contribute to malabsorption.

**Secretion.** The secretory component of rotavirus diarrhea appears to be secondary to virus-induced functional changes at the villus epithelium. The central players in secretion appear to be NSP4 and the ENS. The precise role and targets of secreted NSP4 are unknown. NSP4 may simply amplify the effects of infection in the enterocyte epithelium. However, NSP4 may also act at the crypt epithelium, where it would induce increases in crypt cell [Ca²⁺]i, activate Cl⁻ secretion, and lead to an outflow of water. This Cl⁻ secretion is known to be unrelated to the cAMP-dependent Cl⁻ channel of crypt cells because CFTR-knockout mice are susceptible to rotavirus or NSP4-induced diarrhea (Angel et al., 1998; Morris et al.,1994). The identity of the Cl⁻ channel involved in rotavirus diarrhea in CFTR-knockout mice remains unknown. It is proposed that NSP4 itself may form a channel or that NSP4 activates a dormant Ca²⁺-activated anion channel (Morris et al., 1994). Interestingly, these studies also showed that the age dependence of rotavirus diarrhea is not due to age-dependent expression of a NSP4 receptor or age-dependent Ca²⁺ mobilization but rather to the age dependence of Cl⁻ permeability (Morris et al., 1994). Another possible target of secreted NSP4 is the ENS, which is also a target in classic cholera toxin-induced diarrhea (Lundgren et al., 2003). Indeed, the ENS is rich immediately under the villus epithelium, and it is situated
to receive stimuli from the rotavirus-damaged epithelium. Although NSP4 stimulation of the ENS has not been shown experimentally, it has been shown that the ENS is involved in rotavirus diarrhea (Lundgren et al., 2000). The application of a number of pharmacologic agents that block ENS stimulation (lidocaine, tetrodotoxin, and mecamylamide) significantly lowered the transmembrane potential difference in a virus dose-dependent manner in Ussing chamber or organ bath experiments using rotavirus-infected intestinal tissues. In organ bath experiments, blocking the ENS could change net secretion to net absorption (Lundgren et al., 2003). In live infected animals, repeated administration of lidocaine significantly prevented fluid losses. Thus, it is clear that the ENS is activated during rotavirus infection, and this activation could explain how relatively few infected cells at the villus tips could stimulate crypt cells to secrete electrolytes and water (Lundgren et al., 2003).

While it is unknown if NSP4 directly stimulates the ENS, the ENS is known to respond to a number of molecules released from enterocytes. Cholera toxin induces the release of 5-hydroxytryptamine (5-HT) from enterochromaffin cells in the villus epithelium, and 5-HT is a stimulator of the ENS (Lundgren et al., 2001). It is possible that secreted NSP4 binds to enterochromaffin cells, inducing a release of 5-HT and stimulation of the ENS (Lundgren et al., 2001). Likewise, the secretion of chemokines and prostaglandins by infected enterocytes may serve to stimulate the ENS. Currently, the ENS and NSP4 appear to have the major roles in the secretory response to infection.

**Villus ischemia.** Although damage to the intestinal epithelium is minimal in rotavirus-infected mice, villus ischemia was observed in some studies (Osborne et al., 1988; Starkey et al., 1986). It was proposed that diarrhea could result from virus-induced release of an unknown vasoactive agent from infected epithelium, causing a local villus ischemia and subsequent functional damage to enterocytes (Osborne et al., 1991). However, villus ischemia has not been observed in other animal models, so the significance of this observation remains unknown.

**Intestinal motility.** In some diarrheal infections, intestinal motility is significantly increased. The intestinal transit time is decreased in rotavirus infection, implying increased motility (Michelangeli and Ruiz., 2003). The ENS generally controls motility, but the molecular stimulator of motility is not known. It could be any of the ENS stimulators discussed above. To summarize, rotavirus diarrhea is clearly a multicomponent disease. Good evidence exists for malabsorptive and secretory components. The mediators of these disease components range from primary cellular damage to a secreted viral enterotoxic peptide and a virus-induced interaction with the ENS.

**EPIDEMIOLOGICAL DATA OF ROTAVIRUS G AND P TYPES IN WATER BUFFALOES** - In Italy, buffaloes (Bubalus Bubalis) population consists of approximately 200,000 animals. Buffalo herds are mainly located in the southern regions, where they represent an important economic resource for the industry of milk-derived products. A key role in the etiology of viral infectious diarrhea in buffalo calves has been attributed to rotavirus (Sunil-Chandra and Mahalingam, 1996; Singh and Pandey, 1988). The distribution of P and G genotypes in bovines has been investigated previously (Alfieri et al., 2004; Barreiros et al., 2004; Chang et al., 1996; Falcone et al., 1999; Fukai et al., 1998; Gulati et al., 1999; Isegawa et al., 1993; Ishizaki et al., 1996; Okada and Matsumoto, 2002; Suzuki et al., 1993) showing the prevalence of the VP7 specificities G6, G8 and G10 and of the VP4 specificities P[1], P[5], P[11],
in various combinations and with various distribution. In addition, rare G and P types, G1, G3, G7, G15 and P[17], P[21] have been reported but their epidemiological role in bovines is unknown (Blackhall et al., 1992; El-Attar, 2002; Rao et al., 2000; Isegawa et al., 1994). The detection of bovine-like rotaviruses in humans (Santos and Hoshino, 2005), either with a sporadic or non-sporadic pattern, highlights the potential zoonotic impact of animal rotaviruses for humans and stresses the need for a more in-depth understanding of the epidemiology of animal rotavirus. Early studies on the distribution of the P and G types in buffaloes in Italy reported that RV strains collected in 1993–1994 were P[5],G6, P[11],G8 and P[5],G8 and that strains collected in 1998 were P[1],G8 (Martella et al., 1999), suggesting the epidemiological relevance of G8 rotaviruses. Noteworthy, an unusual G6 buffalo strain, 10733, isolated in 2001, was shown to have the rare P[3] VP4 specificity, 96.2% identical to the VP4 of the reference simian rhesus strain RRV (Martella et al., 2003b), an epidemiological finding apparently unexplainable. The high frequency of identification of G8 strains and the detection of a rare P[3], RRV-like, strain, raise the question as to whether there are epidemiological differences between bovine and buffalo rotaviruses, i.e. whether there is a preferential spread of some rotavirus strains in buffaloes, although bovines and buffaloes are closely- related species and in spite of the not strict rotavirus species-specificity.

In a previous work we have determined the P and G genotypes of group A rotavirus detected in faecal samples from buffalo calves with diarrhea in Southern Italy (Pisanelli et al., 2005). Although buffalo rotavirus has been established as a common agent of diarrhea in buffalo calves (Sunil-Chandra and Mahalingam, 1996; Singh and Pandey, 1988), only a few studies focused on the antigenic/ genetic characterization of buffalo rotaviruses (Martella et al., 1999, 2003b; Gulati et al., 1999). The most common G and P genotype combinations in the buffalo rotaviruses identified in our study were P[5],G6 (57%), followed by P[1],G10 (19%), while P[5],G8 and P[1],G8 strains accounted for 14% and 9.5%, respectively. Previous analyses of ruminant strains collected in Italy in the mid to late 1990s revealed that the P[5],G6 combination was very common in bovines (38.3%), followed by the P[11],G10 combination (31.5%) (Falcone et al., 1999) and that the combinations P[1],G8 and P[5],G8 were present in buffaloes (Martella et al., 1999) but a few P[1] strains were detectable in bovines in the same years (Falcone et al., 1999). Such epidemiological situation seems to be maintained over time in Italy, as analysis of BoRV strains collected between 1999 and 2004 in Italian herds indicates that the P[5],G6 combination is still predominant over P[11],G10 and P[11],G6 (Martella et al., unpublished). Altogether, these data may suggest that there is a predominance in Italy of bovine strains with G6 VP7 and with either P[5] or P[11] VP4 and that the G8 VP7 is rare in bovines but is relatively more common in buffaloes, in association with either the P[5] or P[1] genotype. An interesting finding of our study was the identification of strains displaying the P[1],G10 combination, as such VP4– VP7 combination has not been described in buffalo or bovines previously. Accordingly, it may be hypothesized that the P[1],G10 strain derived from reassortment between a P[1] buffalo strain and a G10 bovine strain. By RNA–RNA hybridization, group A rotaviruses have been classified in a number of genogroups. Members of a genogroup share a high degree of genetic relatedness but have significantly less homology with members of other genogroups. While reassortment between different genogroups is rare, reassortment within a genogroup may occur very frequently (Nakagomi and Nakagomi, 2002). As all bovines rotaviruses belong to a unique genogroup, the finding that some G–P combinations are rare
under natural conditions is of difficult explanation. Particular ecological/management conditions, such as intermingled distribution over the same geographical areas of the buffalo and bovine herds, may have contributed to generation in Italy of such novel, previously unrecognized, P–G combination.

During this study we could analyze rotavirus positive samples from the same herds over a 4-year period, observing a sequential change of the VP4 and VP7 specificities. A P[1],G8 strain, identified in 2001, was replaced by a P[5],G8 strain in 2002 and by a P[5],G6 strain in 2003–2004.

Analogously, a pattern of sequential replacement of the rotavirus antigenic types has been described during a long-term survey in a bovine herd in Italy (Martella et al., 2003c), while a periodic shift of the VP4 and VP7 specificities has been observed in large epidemiological surveys for bovine rotaviruses in Japan (Fukai et al., 2002) and human rotaviruses in Southern America (Timenetsky Mdo et al., 1994) and Australia (Kirkwood et al., 2002, 2003a, 2003b, 2004; Diwakarla et al., 2002), suggesting that a mechanism of antigenic escape drives the antigenic variation of rotaviruses by sequential replacement of the various strains. In conclusion, our data suggests that in Italy buffalo and bovine rotaviruses share similar VP4 and VP7 antigenic specificities, even if G8 rotaviruses seem to be more frequent in buffaloes. Also, a novel reassortant strain, P[1],G10 occurred in buffaloes, presumably by reassortment between a buffalo P[1] strain and a bovine G10 strain, that had never been reported during previous investigations in Italy or elsewhere. Noteworthy, it was not possible to identify strains 10733-like, bearing the rare P[3], RRV-like VP4 (Martella 2003b), puzzling the hypothesis on the origin of such unusual buffalo rotavirus strain detected in 2001 in Southern Italy.

Currently, for prophylaxis of rotavirus neonatal diarrhea of large ruminants at least three vaccines are available in Italy, a killed vaccine, based on a porcinelike strain, P[7],G5, and two live modified vaccines, P[1],G6 and P[5],G6. Continual surveillance for rotavirus genotypes in buffalo herds is required for a better understanding of the global rotavirus ecology and for the optimisation of current vaccines and prevention programs of rotavirus diarrhoea in ruminants.

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