Chapter 1: Introduction

Part of the introductive section of the manuscript is based upon a review in preparation that aimed to gather all information on norovirus risk profiling with a special emphasis on the role of food and animals in norovirus transmission. During a national project, scientists from the Belgian Institute of Public Health and laboratories of food microbiology and animal virology worked closely together in order to develop norovirus detection tools and to collect data on norovirus strains detected in food, animals and humans.

The development of a risk profile on noroviruses in food and potential transmission routes

In preparation

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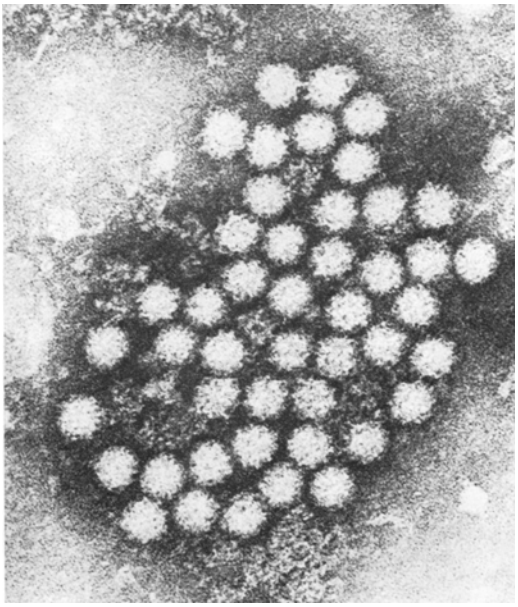

Figure 1: Aggregate of norovirus particles in a stool sample observed by immune electron microscopy (Kapikian et al., 1972).

1.1 Noroviruses

Noroviruses (NoVs) are among the most important causes of both sporadic cases and outbreaks of gastroenteritis in humans of all ages and are responsible for approximately 90% of epidemic non-bacterial outbreaks of gastroenteritis in industrialised countries. Seroprevalence studies indicated that nearly 100% of adults were exposed to one or more NoV infections during the early years of their life (Donaldson *et al.*, 2008). In the Netherlands, the Public Health Institute investigated 941 outbreaks of acute gastroenteritis between 1994 and 2005. Of those outbreaks 6.6% were considered foodborne and 78% were presumptively caused by NoV (Svraka *et al.*, 2007). In the United States, 59% of the yearly reported foodborne illnesses are believed to be caused by viruses and NoVs were linked with 58% of these cases. Also, NoVs were estimated to be the second cause (26%) of hospitalisations after *Salmonella* spp. (35%) (Scallan *et al.*, 2011). In Belgium, since 2007 and for the three subsequent years, NoVs have been identified as the first cause of foodborne gastroenteritis outbreaks before *Salmonella* spp (Baert *et al.*, 2009) (Denayer S., Botteldoorn N. Personal communication). Transmission routes are multiple and can happen through direct contact with shedding persons, contaminated food, sewage-contaminated water, contaminated aerosols and environmental contamination (Siebenga *et al.*, 2010a). NoV infections often occur in large outbreaks in environments where people congregate and where they are frequently associated with high economic impacts (Lee *et al.*, 2011). Outcomes can be particularly severe in health care settings due to the presence of vulnerable patients with underlying severe illnesses (Gustavsson *et al.*, 2011; Schwartz *et al.*, 2011). Besides being an important pathogen in humans, NoVs have been detected in a broad variety of animal species (Martella *et al.*, 2007; 2008; Saif *et al.*, 1980; Wolf *et al.*, 2009; Woode and Bridger, 1978), raising important, yet partly unanswered, questions about the possibility of zoonotic transmissions and the existence of an animal reservoir for NoVs.

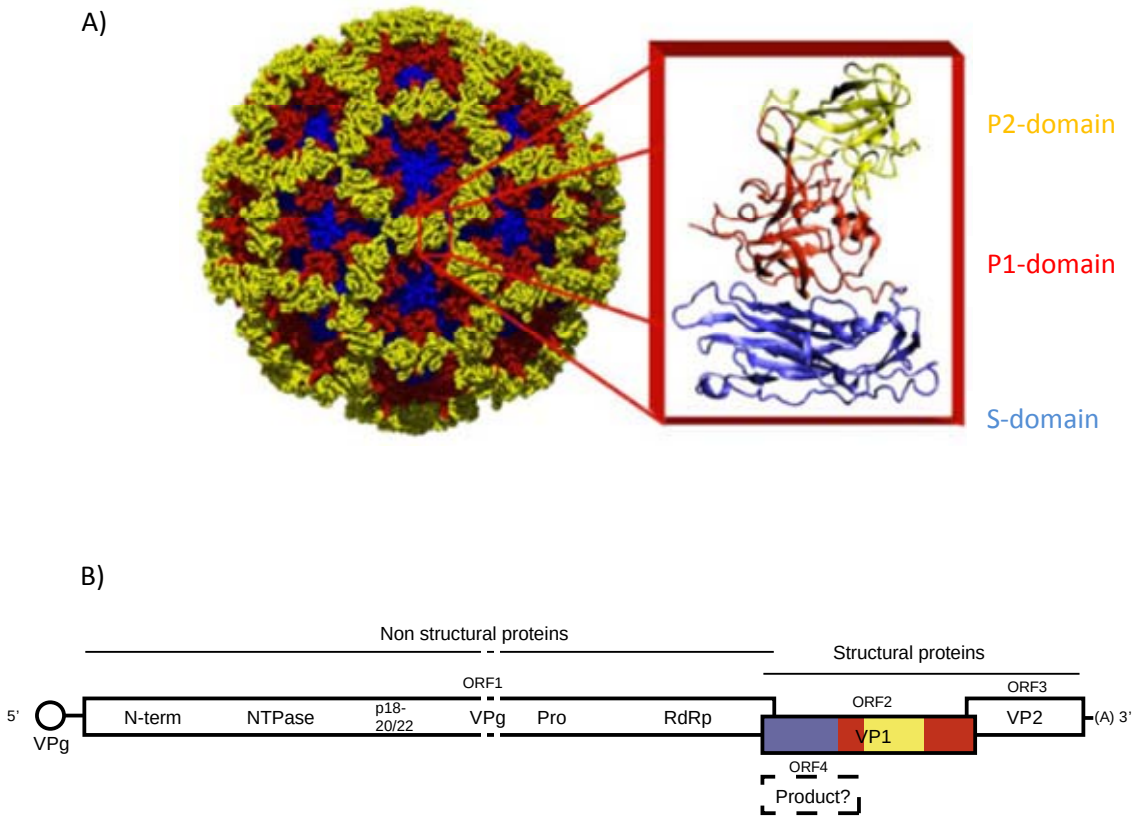

Figure 2: Schematic view of the norovirus viral structure and genome organisation. A: Three-dimensional representation of the norovirus particle with a detailed view of an individual monomer that forms the norovirus capsid. This subunit is composed of the protruding (P) domain (subdivided into sub-domains P1 (in red) and P2 (in blue)) and the shell (S) domain. **B**: Schematic representation of the norovirus genomic organisation. Regions corresponding to the P1, P2 and S domains are highlighted in their respective colors. VPg: genome linked viral protein; N-term: N-terminal protein; NTPase: nucleoside 5'-triphosphatase; Pro: proteinase; RdRp: RNA-dependant RNA-polymerase; VP1: major viral protein; VP2: minor viral protein.

1.1.1 Background

In the late 20's, Zahorsky described the "winter vomiting disease", a disease characterised by diarrhoea and vomiting that typically showed an epidemic peak during the colder months (Zahorsky, 1929). The aetiology of this syndrome was only discovered in 1972 when NoVs (formerly called "Norwalk-like viruses" or "small round structured viruses") were visualised by immune electron microscopy in faeces from volunteers challenged with faecal filtrates from students collected in fall 1968 (Kapikian *et al.*, 1972) (Figure 1). The Norwalk virus GI.1, named after the town of Norwalk (Ohio, USA) where the outbreak took place, remains the prototype strain of the genus Norovirus. The successful cloning of the entire NoV genome in 1990, the obtaining of a full genome nucleotide sequence together with the development of molecular techniques has led to major advances into the understanding of NoV biology and epidemiology (Ando *et al.*, 1995; Jiang *et al.*, 1993). Based on phylogenetic analyses, NoVs were divided into five genogroups that were further subdivided into genotypes reflecting the great diversity that characterises NoVs (Zheng *et al.*, 2006). Still, no standardised classification method is available yet.

Despite numerous efforts, human NoV (HuNoV) does not dispose of an efficient *in vitro* cell culture system or a small animal model for the study of virus replication and pathogenesis (Duizer *et al.*, 2004; Lay *et al.*, 2010). A 3-dimensionnal model of human small intestinal cells has been reported but still needs to be confirmed (Straub *et al.*, 2007). The use of genetically and antigenically equivalent virus-like particles (VLPs) has allowed the study of host-virus interactions and immune responses; VLPs were also abundantly used for prophylactic or diagnostic purposes (Mauroy *et al.*, 2009a; Shirato *et al.*, 2008; Zhang *et al.*, 2006). The murine NoV (MNV) was found to propagate and form plaques in RAW 264.7 cells, an immortalised mouse macrophage and dendritic cell line and constitutes up to date the only efficient cell culture system for NoVs (Karst *et al.*, 2003). Moreover, the murine model offers the advantage of being an affordable model for *in vivo* experimentation. Thus, the MNV is nowadays considered as the most suitable surrogate for NoV studies in the absence of an efficient replication alternative for HuNoVs.

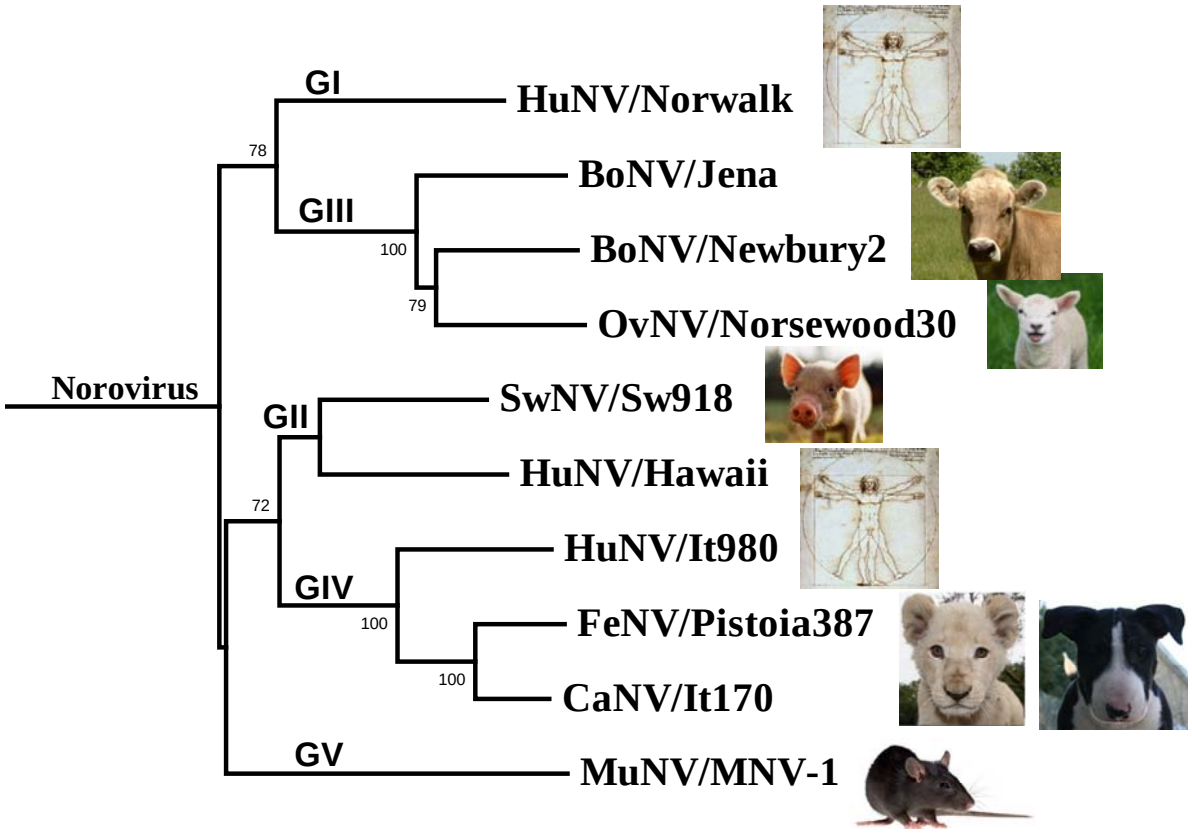

Figure 3: Phylogenetic analysis of the genogroups that compose the *Norovirus* genus. A multiple alignment of partial capsid gene sequences was conducted using ClustalW for the norovirus reference strains. Numbers situated at branches indicate bootstrap values. GenBank accession numbers are as follows: Norwalk M87661; Hawaii U07611; It980 FM865412; Jena AJ011099; Newbury2 AF097917; Norsewood30 EU193658; Sw918 AB074893; Pistoia387 EF450827; It170 EU224456; MNV-1 AY228235. HuNV: human norovirus; BoNV: bovine norovirus; OvNV: ovine norovirus; SwNV: swine norovirus; FeNV: feline norovirus; CaNV: canine norovirus; MuNV: murine norovirus.

1.1.2 Viral structure and genomic organisation

NoV virions are small non-enveloped spherical particles of approximately 28-32 nm in diameter. The 3-dimensional structure of recombinant viral capsids analysed by crystallography revealed a T=3 icosahedral symmetry with 180 molecules of the major capsid protein (VP1) organised into 90 dimeric capsomers (Prasad *et al.*, 1994) (Figure 2A). In total, three proteins are found in mature NoV virions: major and minor capsid proteins (VP1 and VP2, respectively) and a genome-linked virus protein (VPg). VPg is covalently linked to the 5' end of the genomic and subgenomic RNA and is likely to play an important role in the initiation of RNA translation (Daughenbaugh *et al.*, 2003; 2006).

NoVs are non-enveloped viruses possessing a single-stranded, positive-sense, polyadenylated RNA genome of about 7500 nucleotides (nt) in length (Green, 2007) (Figure 2B). Three overlapping open reading frames (ORFs) encode the non-structural (ORF1) and structural (ORF2 and ORF3) viral proteins. The ORF1-encoded polyprotein is cleaved by the viral proteinase (Pro) into six mature products with as gene order: the N-terminal protein (N-term), NTPase, picornavirus 3A-like protein, VPg, proteinase and RNA-dependent RNA polymerase (RdRp) (Green, 2007; Sosnovtsev *et al.*, 2006). Due to the high conservation in the latter region, the polymerase has been a widely used target for molecular detection assays (Jiang *et al.*, 1999b; Vennema *et al.*, 2002). ORF2 encodes the VP1 that contains an N-terminal arm, a shell or S-domain and a protrusion or P-domain (Figure 2B). The P-domain is further divided into 2 sub-domains called P1 and P2, the latter corresponding to the most variable region of the capsid. The hypervariable stretch within P2 is thought to play an important role in receptor binding and immune reactivity (Hardy, 2005). Finally, ORF3 encodes the VP2, a small protein of unknown function present in only 1 or 2 copies per virion. A fourth ORF overlapping the 5' end of the VP1 coding sequence in a different reading frame was described for MNVs (Thackray *et al.*, 2007). This ORF was predicted to encode a 214-amino-acid protein for which no function has been proposed yet.

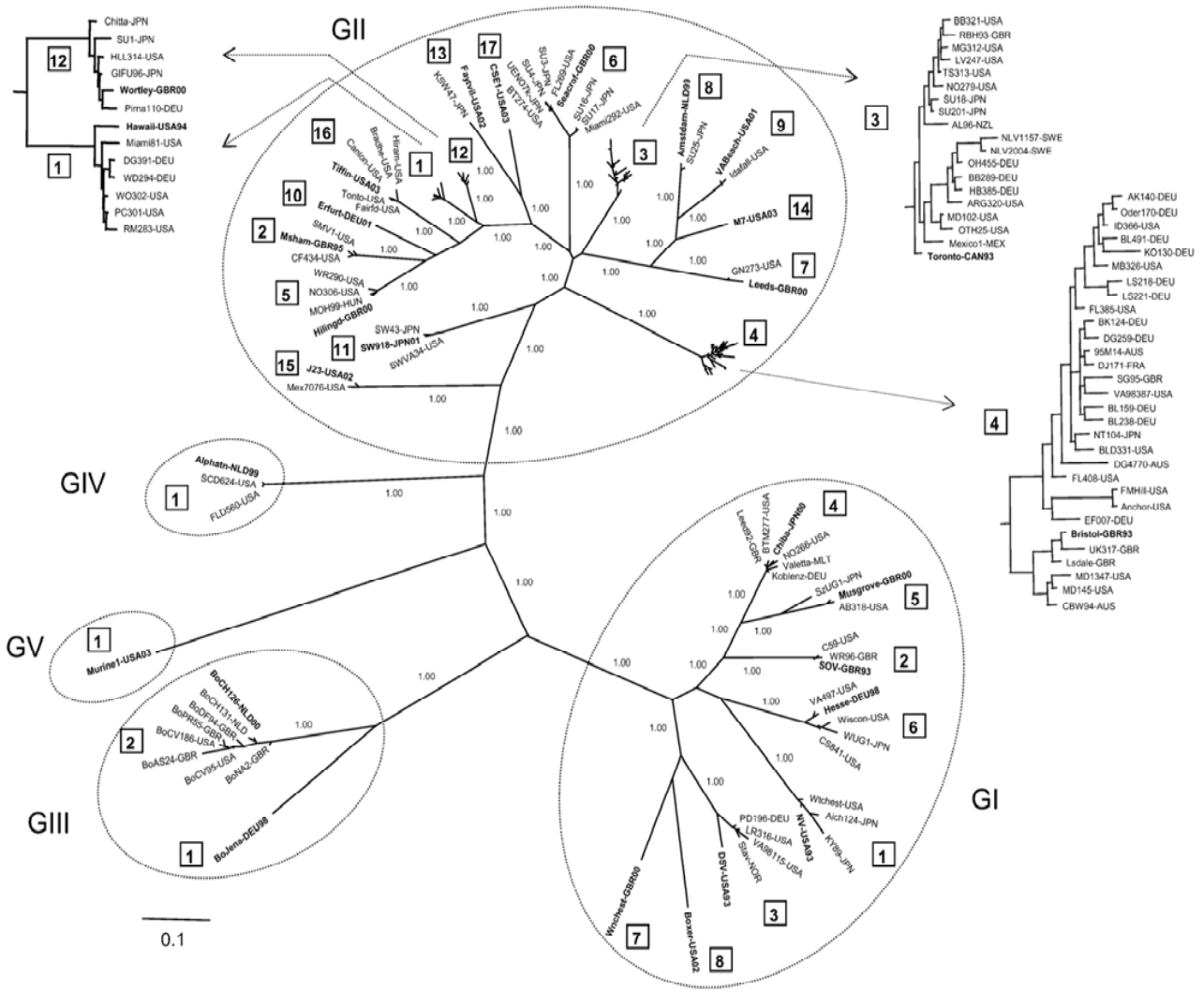

Figure 4: Phylogenetic analysis of complete capsid amino acids sequences of 141 norovirus strains (Zheng et al., 2006)

1.1.3 Taxonomy and classification

As proposed by the *International Committee on Taxonomy of Viruses* (ICTV), *Norovirus* belongs to the *Caliciviridae* family along with genus *Lagovirus*, *Nebovirus*, *Vesivirus* and *Sapovirus*. NoVs are genetically highly diverse with approximately 46% nucleotide divergence in the capsid gene region between its five genogroups (GI–V) (Zheng *et al.*, 2006). HuNoVs belong to genogroups I, II and IV whereas GIII and GV enclose bovine NoVs (BoNoVs) and MNVs, respectively. Porcine NoVs (PoNoVs) are genetically close to human NoV and belong to GII. Recently, NoV sequences that cluster within GIV have been detected in diarrheic faeces in a lion cup and dogs (Martella *et al.*, 2007; Martella *et al.*, 2008; Mesquita *et al.*, 2010). Furthermore, NoVs have been detected in sheep and were shown to cluster within GIII (Wolf *et al.*, 2009) (Figure 3). Despite multiple efforts, there is still no internationally recognised nomenclature system available for NoVs hampering the comparison between molecular data from epidemiological studies in different parts of the world. The Noronet network, a consortium of scientists from all over the globe sharing virological, epidemiological and molecular data on NoV, aims to design a well founded standardised nomenclature for existing and emerging NoV genotypes and variants or sub-lineages and the development of a publicly available genotyping tool (Kroneman *et al.*, 2011). These tools will allow scientist to talk a common language for early recognition of globally emerging strains or indications of common sources. Awaiting an official classification system, a commonly excepted nomenclature based on the diversity of complete amino acid sequences of the VP1 gene divides NoVs into genogroups, genotypes and even subvariants for GII.4 NoVs with uncorrected pairwise differences ranging between 44.9-61.4 %, 14.3-43.8 % and 5-14%, respectively (Zheng *et al.*, 2006; 2010) (Figure 4). They suggested the division of the five genogroups into 29 genotypes: eight in GI (GI.1 to GI.8), seventeen in GII (GII.1 to GII.17), two in GIII (GIII.1 and GIII.2), one in GIV and one in GV. Further phylogenetic analyses, divided PoNoV into 3 genotypes: GII.11, GII.18 and GII.19 (Wang *et al.*, 2005; 2007), raising GII genotypes to the number of 19. Although biologically highly diverse, all MNVs cluster into one single genogroup with limited sequence divergence (Thackray *et al.*, 2007).

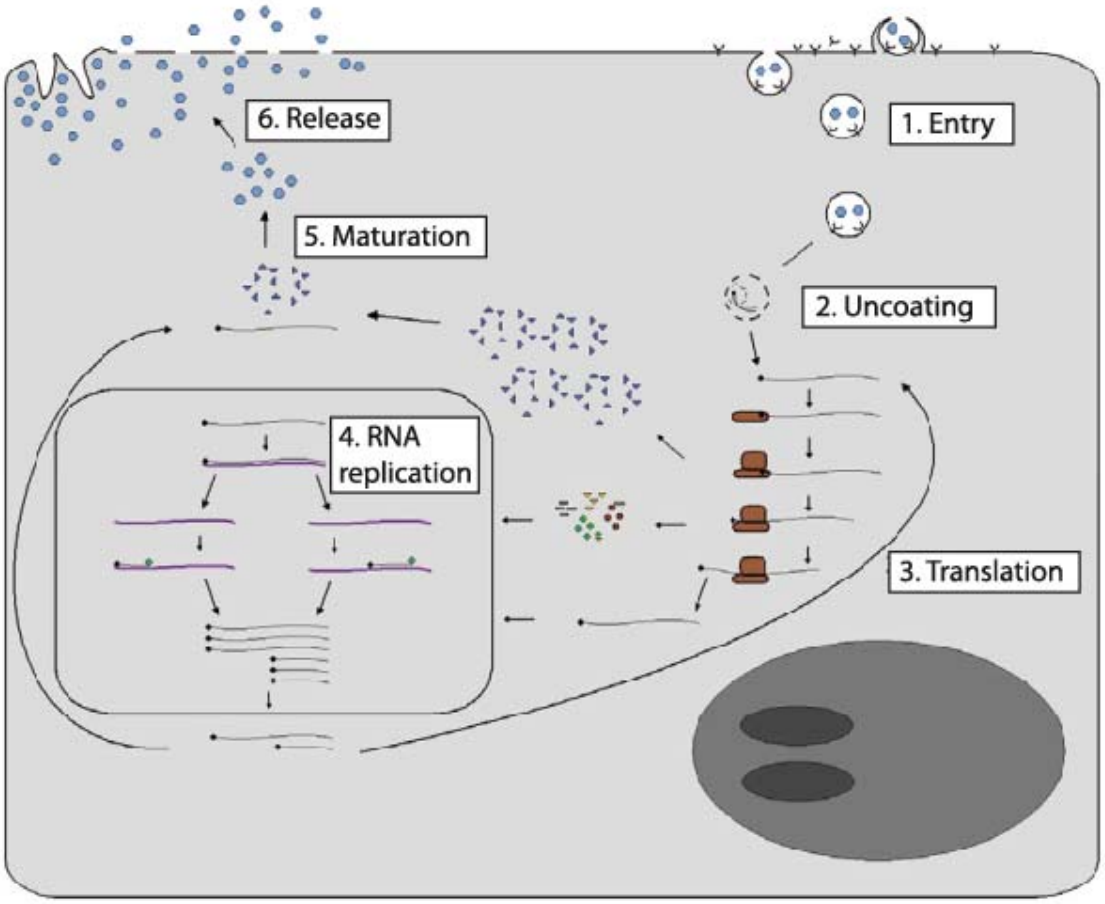

Figure 5: Schematic representation of the proposed replication strategy for the caliciviruses including noroviruses. Similarly to other positive-strand RNA viruses, the replication cycle is divided into the following steps: (1) Entry; (2) Uncoating; (3) Translation; (4) RNA replication; (5) Maturation; and (6) Release. (Adapted from Green et al., 2007)

1.1.4 Replication cycle

Little is known regarding NoV replication but the strategy is believed to resemble that described for other members of the *Calicivirdae* family sharing a lot of characteristics with other positive strand RNA viruses (Figure 5). After the interaction of the virion with the host cell (likely by the recognition of a virus specific-receptor), the particle will enter into the cell and uncoat to release its RNA genome in the cytoplasm. This interaction is suspected to rely upon an essential receptor recognition believed to involve cellular carbohydrates (Rademacher *et al.*, 2008; Tan and Jiang, 2010). Experimental data showed that MNV, similarly to feline calicivirus, can use terminal sialic acids on gangliosides as attachment receptors during binding to murine macrophages (Taube *et al.*, 2009). Feline calicivirus was shown to depend on a low pH step during entry, a feature that was not found for MNVs that enter the cell in a pH-independent way (Perry *et al.*, 2009). Protein-RNA interactions between the viral VPg protein and the cellular translation initiation factors (eIFs) including eIF4E were found essential for the initiation of translation of the incoming mRNA (Daughenbaugh *et al.*, 2003; Daughenbaugh *et al.*, 2006). The ORF1 in translated to produce the nonstructural polyprotein further on cleaved into several precursors and products by the viral proteinase (Someya and Takeda, 2009; Sosnovtsev *et al.*, 2006). For RNA replication, the initiation of the synthesis of negative strand RNA from the genomic RNA (gRNA) template occurs at the 3’ end of the positive strand RNA probably enhanced by the interaction with cellular proteins. Once synthesised, the negative strand RNA will serve as template for the transcription of both full-length gRNA and subgenomic RNA (sgRNA). These transcription reactions are catalysed by the RNA-dependent RNA-polymerase encoded by the viral polyprotein. The abundant synthesis of sgRNA in comparison with gRNA serves as bicistronic template for the synthesis of the two structural proteins VP1 and VP2. Like all positive sense RNA viruses, MNV RNA replication was intimately associated virus-induced membrane rearrangements that comprised of a collection of cytoplasmic vesicles, components of the endocytic and secretory pathway (endoplasmic reticulum, the Golgi apparatus and endosomes) (Hyde *et al.*, 2009). The final steps of the viral replication strategy (RNA packaging, capsid maturation and release) are still poorly understood.

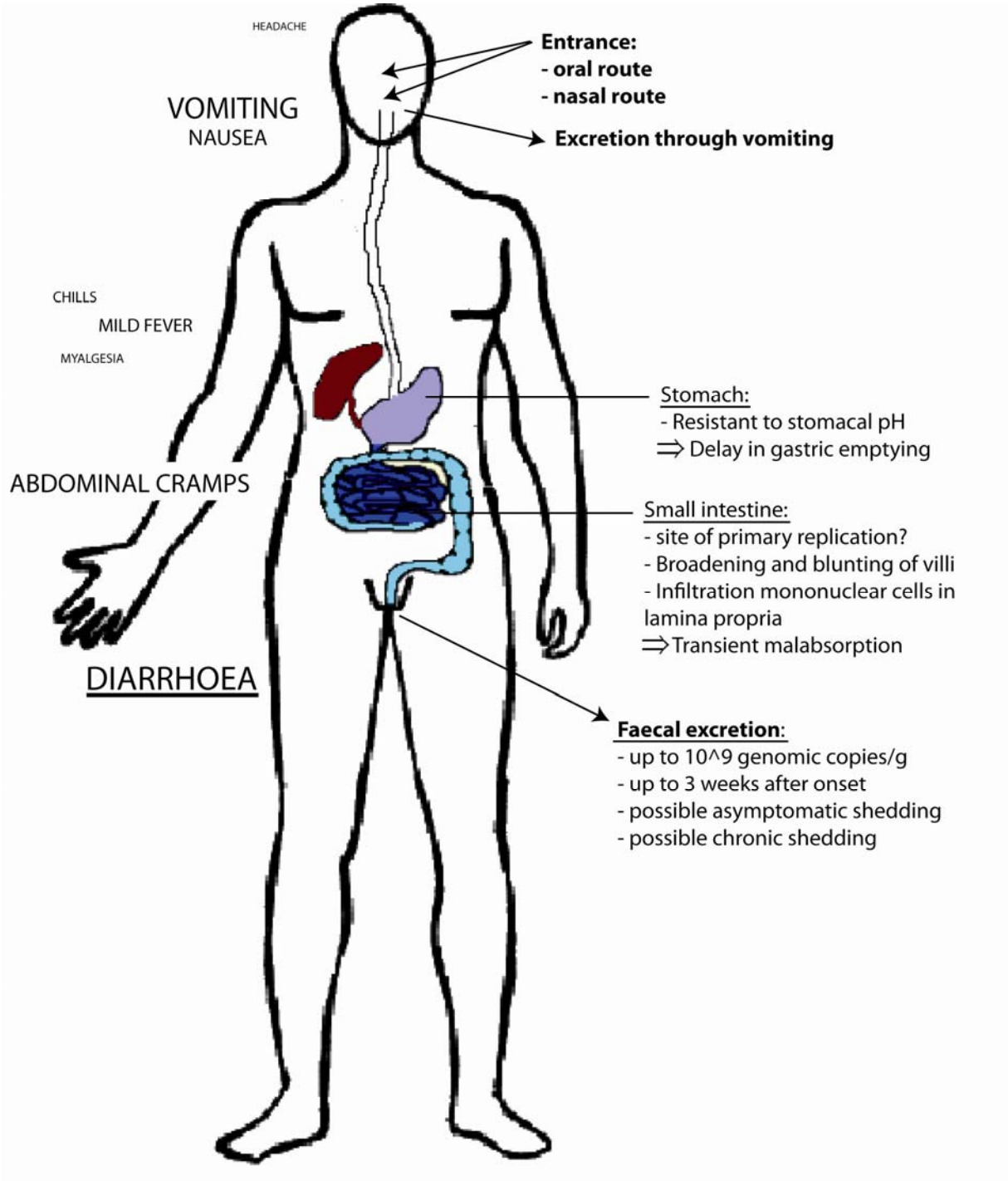

Figure 6: Pathogenesis and clinical aspects of norovirus infections in humans. Size of police in which the symptoms are given is proportional to the probability of occurrence during norovirus infection.

1.1.5 Norovirus infections in humans and animals

In Humans

In humans, NoVs are an important cause of acute gastro-enteritis in both children and adults. NoV infection symptoms are in order of importance: non haemorrhagic diarrhoea, vomiting, abdominal pain/cramps, nausea, mild fever, chills, myalgia, and headaches (Gallimore *et al.*, 2004; Rockx *et al.*, 2002) but asymptomatic infections have been experimentally observed in volunteer studies (Graham *et al.*, 1994) (Figure 6). Despite the acute onset of illness (within 24 to 48 hours), clinical signs are mild and self-limiting, lasting for 24 to 48 hours after their appearance. Consequently, medical assistance is rarely needed with the exception of more vulnerable populations such as juvenile, elderly or immunocompromised patients, for which more intensive care can be required. In the absence of specific therapeutic measures, treatment is focused on providing supportive care like rehydration. Chronic infections have been described in patients with underlying illnesses responsible for impaired immunity e.g. acquired immune deficiency syndrome (AIDS) patients or transplant recipients (Goller *et al.*, 2004; Lee *et al.*, 2008; Sukhrie *et al.*, 2010). These infections come along with chronic NoV shedding for periods up to several months or even years. Together with asymptomatic carriers, chronic shedders may constitute an important reservoir for NoVs. The lack of an easy reproducible cell culture system together with the absence of a small animal model for HuNoVs is responsible for the scare information on the pathogenesis of this pathogen.

Up to date, the majority of the data available have been gathered from experimental infections of volunteers in the early years of its discovery. NoV infections are limited to the upper part of the digestive system even though detections of NoV RNA in sera or cerebrospinal fluid of infected individual suggest that NoV could spread to peripheral tissues (Ito *et al.*, 2006; Takanashi *et al.*, 2009). Histological lesions are localised in the mucosal layer of the proximal part of the small intestine with broadening and blunting of intestinal villi, epithelial cell disarray, crypt cell hyperplasia, enterocyte cytoplasmic vacuolisation, and infiltration of inflammatory cells into the lamina propria (Agus *et al.*, 1973; Troeger *et al.*, 2008).

In the absence of virus neutralisation methods, no actual serotypes could be defined for NoV but the high genetic diversity could correspond to antigenic diversity explaining the absence of cross-protective responses when infected by NoV of different genotypes. Moreover, strain specific and protective immunity following infection was shown to be short-lived (Matsui and Greenberg, 2000). Together these features are in line with the fact that one can suffer from repeated NoV infections from childhood to old age. Although NoVs causes gastro-enteritis in all ages, differences in susceptibility to infection were observed among volunteers submitted to identical virus challenge protocols suggesting the existence of intrinsic host-factors of resistance based on the presence or absence of virus receptors at the surface of the host cells (Gary *et al.*, 1987; Parrino *et al.*, 1977; Thorven *et al.*, 2005). Recent evidence shows that susceptibility or resistance is given by the virus ability to bind to the polymorphic human histo-blood group antigens (HBGAs) including ABO blood groups, H and Lewis antigens (Ruvoen-Clouet *et al.*, 2000; Tan and Jiang, 2010). HBGAs are complex carbohydrates linked to glycoproteins or glycolipids at the surface of red blood cells and epithelial cells of tissues in contact with the external environment that can be used by viruses as receptor for binding or attachment. Individuals resistant to infection seem to lack the necessary carbohydrates required this virus-host interaction but a diversity of binding patterns between NoV strains have been found due to their genetic variability (Le Pendu, 2004).

In animals

Shortly after the detection of NoV in human clinical samples, related viruses were identified in cattle and pigs (Saif *et al.*, 1980; Woode and Bridger, 1978) and grouped into the NoV genus. Recently, viruses newly detected in the murine, ovine, feline and canine species have been added to the NoV genus (Karst *et al.*, 2003; Martella *et al.*, 2007; 2008; Wolf *et al.*, 2009). Regardless of MNVs, little information is available on the clinical significance of animal NoVs as they were either detected in faeces from healthy individuals (pigs and sheep) (Scipioni *et al.*, 2008a; Sugieda and Nakajima, 2002; Wolf *et al.*, 2009) or found in association with other enteric pathogens that could explain the illness (cattle, lion cup and dogs) (Martella *et al.*, 2007; 2008; Mauroy *et al.*, 2009a). Young pigs, calves and non-human primates have been successfully infected in experimental conditions with HuNoV for the study of NoV biology in the absence of a small animal model (Bok *et al.*, 2011; Cheetham *et al.*, 2006; Souza *et al.*, 2007; 2008).

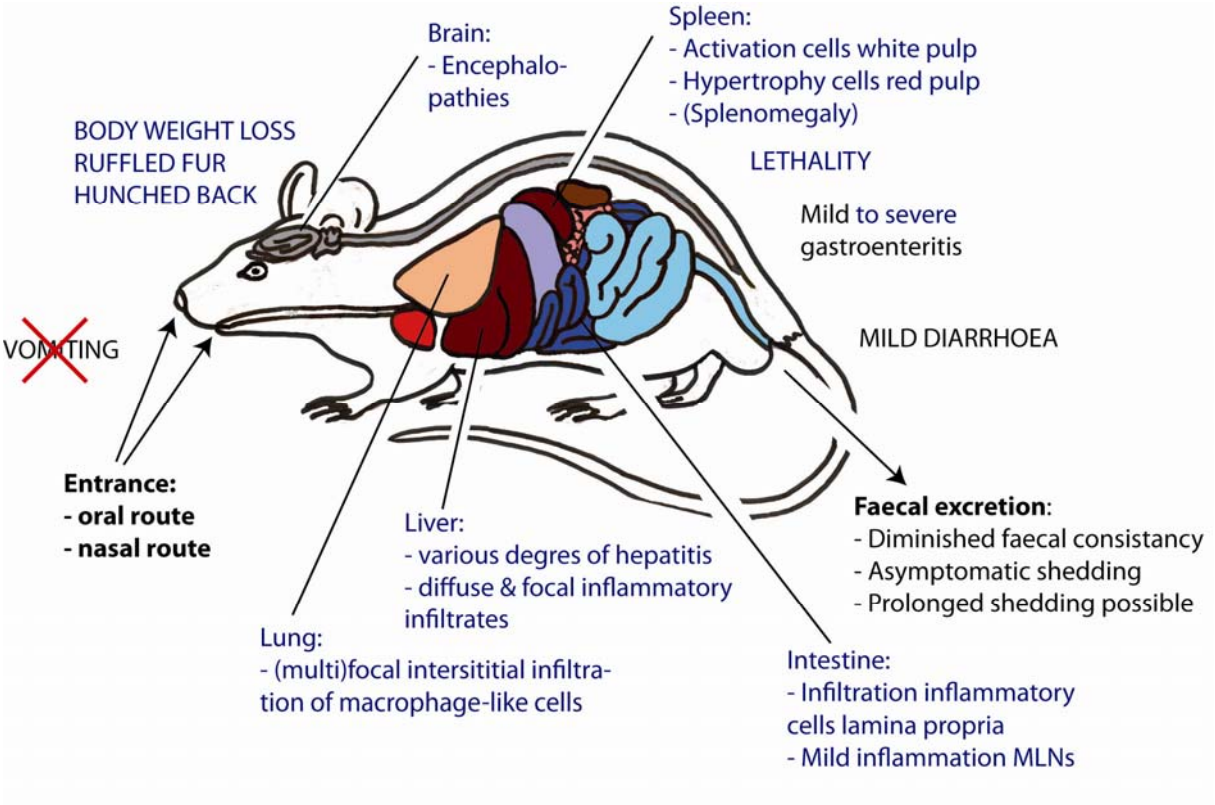

Figure 7: Pathogenesis and clinical aspects of norovirus infections in mice. Text written in blue corresponds to lesions and clinical signs restricted to immunocompromised mice.
MLN: mesenteric lymph nodes.

A NoV causing sporadic deaths and multisystemic disease was first described in 2003 in severly immunocompromised laboratory mice and was referred to as MNV-1 (Karst *et al.*, 2003). The illness was characterised by encephalitits, cerebral vasculitis, meningitis, hepatitis and pneumonia (Karst *et al.*, 2003; Ward *et al.*, 2006). MNV-1 was found to propagate and form plaques in RAW 264.7 cells, an immortalised mouse macrophage and dendritic cell line and constitutes up to date the only efficient cell culture system for NoVs (Wobus *et al.*, 2004). Moreover, MNV-1 is infectious when inoculated by peroral or intranasal route and spreads naturally between immunocompetent mice (Hsu *et al.*, 2005; Mumphrey *et al.*, 2007). Thus, the murine model offers the advantage of being an affordable model for *in vivo* experimentation (Wobus *et al.*, 2006). MNVs were shown to be one of the most prevalent pathogens in research mice (Mähler et Köhl, 2009; Kitajima et al., 2009), seroprevalence values of up to 20% have been found in research colonies (Hsu *et al.*, 2005). Since the first description of MNV-1, over 35 new MNV isolates have been identified exhibiting biological diversity (Hsu *et al.*, 2007; Thackray *et al.*, 2007).

MNVs infect myeloid cells *in vitro* namely a variety of macrophage (Mφ) and dendritic (DC) cell lines. MNV-1 may also infect myeloid cells *in vivo.* This is supported by the detection of viral non-structural proteins in the lamina propria of rare intestinal villi (Mumphrey *et al.*, 2007) and MNV-1 antigen in cells resembling to Mφ in the liver, and Mφ and DCs in the spleen (Wobus *et al.*, 2004). Nevertheless, this *in vivo* cellular tropism still needs to be confirmed. The course of MNV-1 infection in wild-type mice is rapid with viral titres peaking at 1-3 days post-infection (dpi) in organs like intestine, mesenteric lymph nodes (MLNs), liver and spleen (Liu *et al.*, 2009; Mumphrey *et al.*, 2007). A peroral inoculation dose as low as 10 plaque forming units (pfu) was able to cause a low-level infection in part of the inoculated mice with detectable virus loads in intestines, spleen and MLNs (Liu *et al.*, 2009). MNV-1 infections in immunocompetent mice are limited to mild intestinal inflammation accompanied by diminished faecal consistency in a majority of infected animals (Liu *et al.*, 2009; Mumphrey *et al.*, 2007). It is remarkable for an enteric virus that MNV-1 infectious particles can be found in peripheral tissues of infected wild-type mice. This property was also observed for other naturally infecting MNVs UM2, UM3, and UM4 (Hsu *et al.*, 2006) but data is still lacking for other MNVs. Persistent infections have been described for nearly all reported MNVs both in immunocompromised and immunocompetent mice with detectable infectious particles in tissues for weeks or even months after the infection (Wobus *et al.*, 2006). Most experimental data rely on MNV-1 studies that have been conducted under

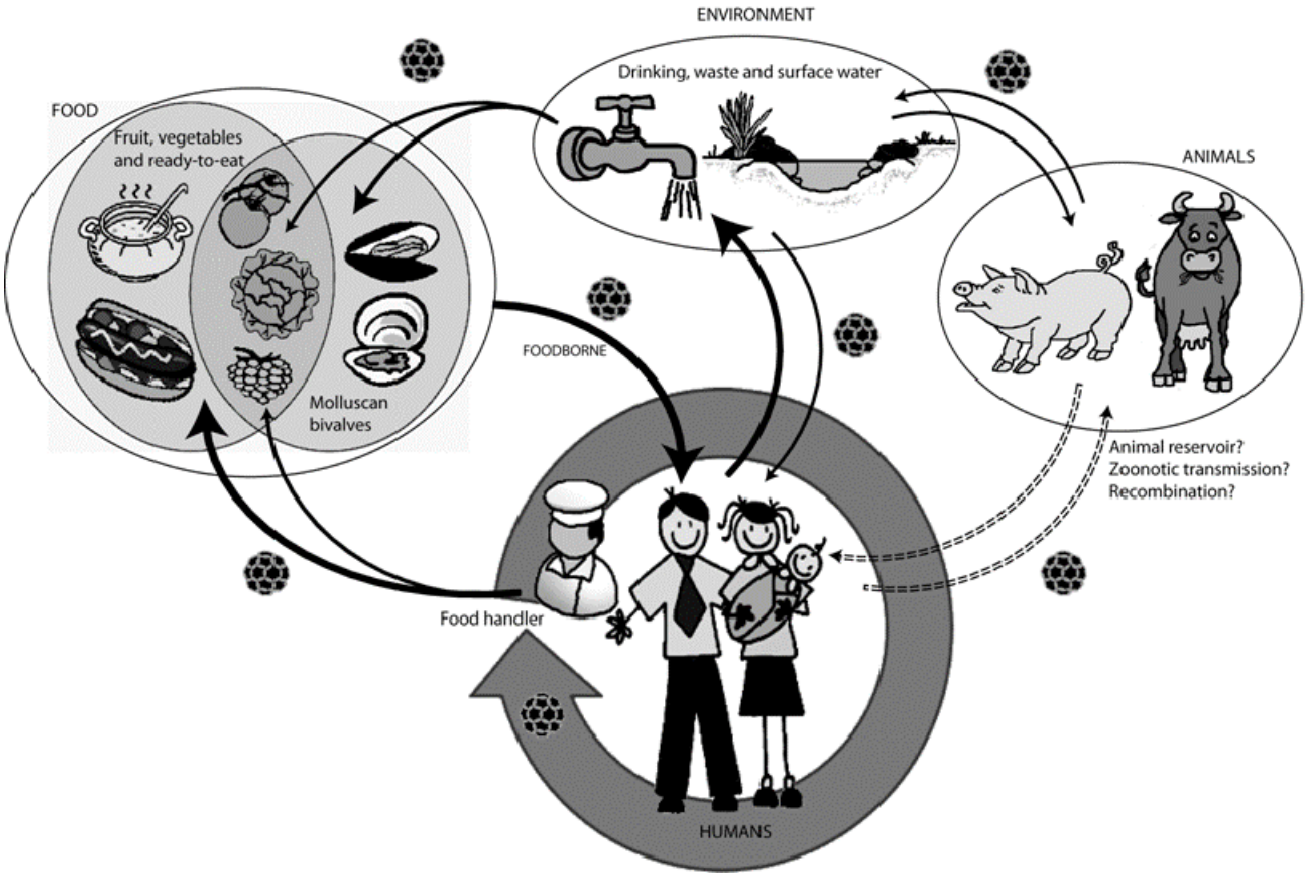

Figure 8: Schematic overview of the transmission routes of norovirus. Solid and dashed arrows indicate proven and hypothetical transmission routes, respectively. The thickness of the arrows is related to the likeliness of the transmission route.

disperse conditions for: (i) the inoculation dose, (ii) the immunological status of infected mice, (iii) organs analysed, (iv) duration of the experiments and (v) the detection methods rending the comparison extremely delicate. The main findings known for MNVs infecting immunocompetent or immunodeficient mice are schematised in figure 7.

1.1.6 Epidemiology, transmission and zoonotic potential

Faecal-oral spread by direct contact between individuals is the major mode of transmission for NoVs (Kroneman *et al.*, 2008a), although other transmission routes exist. A schematic overview of proven and hypothetical transmission routes is shown in figure 8. Humans are infected by NoVs through faecal-oral (or vomitus-oral route) route either by direct contact with an infected individual or via faecally contaminated vehicles, e.g. food, water or surfaces. The spread of NoVs contact with contaminated surfaces or infected persons, is facilitated by a number of factors such as (i) the low infectious dose of 10 to 100 infectious virus particles (Teunis et al., 2008), (ii) the prolonged duration of viral shedding, even after resolving of the symptoms, (iii) the existence of asymptomatic NoV infections, (iv) the stability of the virus in relatively high concentrations of chlorine and at a wide range of temperatures (from freezing to 60°C) and (v) the lack of complete cross-protection against the diverse NoV strains and an inadequate long-term immunity. These characteristics make it particularly difficult to control the propagation of the infection in closed institutions such as hospitals, health care centres and elderly homes. Consequently, outbreaks can be associated with high economic impacts if entire wards are to be closed (Koopmans, 2009; Lee *et al.*, 2011). NoVs can also be particular troublemakers in the leisure industry where large outbreaks have been repeatedly reported on cruise ships, in holiday resorts and youth camps (Dahl, 2006; Migliorati *et al.*, 2008; ter Waarbeek *et al.*, 2010; Verhoef *et al.*, 2008).

Food-borne transmissions have been estimated to account for 14% of infections due to NoV (Lopman *et al.*, 2003). Considering only the outbreaks for which sufficient epidemiological information was available, 10% were reported as foodborne, 2% as waterborne, and the rest as person-to-person outbreaks reported to the Foodborne Viruses in Europe (FBVE) network between 1990 and 2008 (Kroneman *et al.*, 2008a). Food can be contaminated after: (i) contact with faeces or faecally contaminated water; faecally soiled materials including hands, (ii)

contact with vomit or water contaminated by vomit, (iii) contact with contaminated environments and (iv) aerosols generated by infected people (Koopmans and Duizer, 2004). Consistently, foodhandlers have been identified as central to this issue and were frequently designated as the source of the food contamination at any moment along the food chain (Baert *et al.*, 2009; De Wit *et al.*, 2007). Several food stuffs have been preferentially implicated as vehicles of transmission in outbreaks such as minimally-processed fruits and vegetables, deli foods and shellfish (Baert *et al.*, 2009; Sivapalasingam *et al.*, 2004; Stals *et al.*, 2011; Webby *et al.*, 2007). Bivalve shellfish are notorious as a source of foodborne viral infections and are responsible for large, occasionally international, outbreaks (Le Guyader *et al.*, 2006). A first reason why shellfish are at risk food is the fact that they are frequently consumed uncooked or only with a light heat treatment which is not sufficient to guarantee viral safety of the prepared shellfish (Croci *et al.*, 1999). A second motive for this consideration is the ability of shellfish to filter large volumes of water as part of their feeding activities (up to 24 l of water/h), enabling the accumulation and concentration of viral NoV particles in the digestive glands (Le Guyader *et al.*, 2009). The key role of the foodhandler in NoV foodborne transmission led to the conclusion that person-to-person transmissions constitute the main transmission route for NoVs.

Genotyping and the molecular characterisation of NoV strains detected in different matrices are both crucial for the understanding of NoV transmission routes and the identification of common-source outbreaks. Although real-time reverse transcriptase (RT)-PCR products could be used for sequencing, conventional (nested) RT-PCR still remains a powerful tool for virus characterisation as it enables the amplification of longer sequences that can be compared to published NoV sequences. Different regions in the polymerase gene (regions A and B) and the gene encoding the major capsid protein VP1 (regions C, D, and E) (Figure 9) (Anderson et al., 2003; Ando et al., 2000; Gonin and Couillard, 2000; Kojima et al., 2002; Noel et al., 1997; Vennema et al., 2002; Vinje et al., 2004) have been targeted for NoV genotyping but the use of distinct regions has resulted in scattered data and difficulties in comparing the results from different studies (Vinje et al., 2003).

Despite the great genomic variability between human GII NoVs, GII.4 has been by far the most detected genotype being responsible for 60 to 70% of the outbreaks reported to the FBVE between January 2002 and January 2007 (Kroneman *et al.*, 2008a; 2008b). During this period, 4 large pandemics (1995-1996, 2002, 2004-2005 and 2006) have been identified

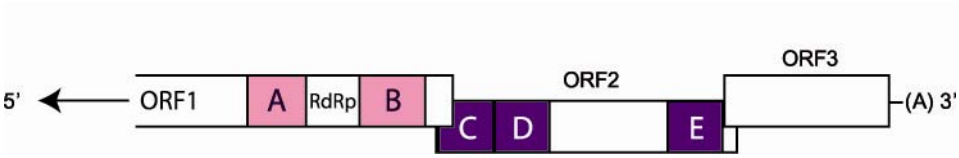

Figure 9: Schematic representation of the locations of the genomic regions used for norovirus genotyping. Adapted from Vinjé *et al*., 2004. RdRp: RNA-dependent RNA polymerase.

corresponding each time to the emergence of one or two new variants of the GII.4 lineage and the displacement of the previously predominant circulating ones (Bull *et al.*, 2006; Lopman *et al.*, 2004; Siebenga *et al.*, 2009; Tu *et al.*, 2008). NoV epidemiology seems to mirror that of influenza A viruses. Thus, NoV activity is associated with the emergence of novel GII.4 variant each two to three years. The emergence of these new epidemic NoV subvariants was linked with the accumulation of mutations in the P2 subdomain of the capsid allowing antigenic escape from host immune responses (Lindesmith *et al.*, 2011; Lindesmith *et al.*, 2008). Similarly the higher epidemiological fitness of the GII.4 lineage was attributed to a higher rate of evolution of the virus capsid proteins (Bull *et al.*, 2010) that could be influenced by four major factors: (i) host receptor recognition, (ii) sequence space, (iii) duration of human herd immunity, and (iv) replication fidelity (Bull and White, 2011).

NoVs have been detected in animals in close interaction with humans like cattle, pigs and dogs (Scipioni *et al.*, 2008a). There is no proof that animal contact, directly or indirectly, can be a source of NoV infection but the detection of related NoVs in animal species in close contact with human beings raises suspicion for cross-species or zoonotic transmissions and the existence of an animal reservoir for NoVs. Therefore, NoV prevalence studies have been realised in order to understand the role of domestic animals in the NoV transmission routes. In Belgium, results for the apparent molecular presence of BoNoVs and PoNoVs were in line with previously published studies and suggested that NoVs are endemic in Western Europe (Mauroy *et al.*, 2008; 2009a; 2009b). The clinical impact of BoNoVs and PoNoVs still remains unclear as few experimental data is available, for some survey studies no clinical data was available and for the majority of positive samples the signs of gastroenteritis could be associated to other pathogens (Mauroy *et al.*, 2009a; 2009b). This is true for all other animal NoVs detected until now (Bank-Wolf *et al.*, 2010; Martella *et al.*, 2008) with the exception of murine NoVs that cause a fatal systemic disease in immunocompromised mice (Karst *et al.*, 2003). So far, all sequences obtained from human clinical samples clustered with human NoV sequences and most NoVs detected in animals, although related to human NoVs, clustered into genotypes or genogroups proper to each species. Although NoVs are considered to be host-species specific and no zoonotic transmissions have been evidenced yet, it mustn’t be rules out that animals could still act as reservoirs for NoVs transmissions or host viral recombination events leading to new emerging strains.

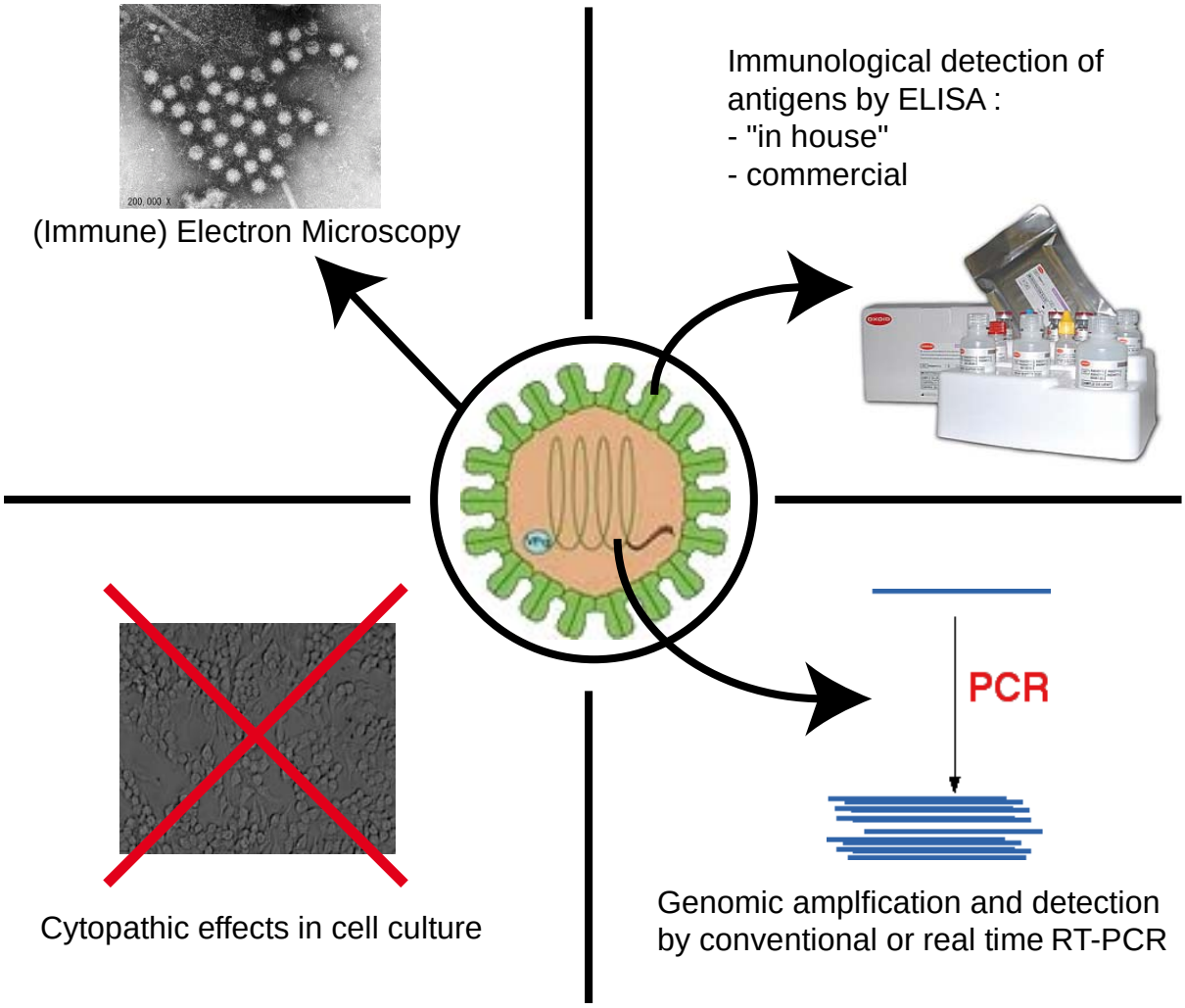

Figure 10: Laboratory diagnosis strategies for the detection of noroviruses.

1.1.7 Diagnosis of norovirus infections

Presumptive diagnosis of NoV infection can be done during an outbreak based on the Kaplan-criteria: (i) no bacteria or parasites agent is found; (ii) mean (or median) duration of illness of 12 to 60 hours; (iii) mean or (median) incubation period of 24 to 48 hours; and (iv) vomiting occurs in more than half of cases (Kaplan *et al.*, 1982). However, the criteria lack sensitivity and about 30 % of norovirus outbreaks do not meet these criteria. Consequently, a viral aetiology should not be excluded if the criteria are not met and any diagnosis based on clinical signs should be further confirmed in the laboratory.

Laboratory diagnosis methods for NoV infections rely on (i) virus particle observation by electron microscopy, (ii) antigen identification by immunological assays or (iii) genomic amplification by RT-PCR and are schematised in figure 10. After numerous vain efforts (Duizer *et al.*, 2004; Lay *et al.*, 2010; Leung *et al.*, 2010), the development of an easily reproducible cell culture system for HuNoVs has made little progress. A major drawback for the use of immunological and molecular methods is the fact that NoVs are genetically and antigenetically highly diverse. The latter feature entails no detection method can detect all NoV at the same time and multiplexing is crucial to broaden this spectrum.

Electron microscopy (EM). Historically, EM allowed the first identification of NoVs. Allowing the direct visualisation of the virus particles, this method is particularly advantageous for the identification of yet undiscovered NoVs. EM remains a valuable diagnostic tool especially for its rapidity but this technique requires the presence high viral loads in the analysed sample. Although sensitivity can be improved by the technique of immune-EM, this tool shows its limits for the use in routine laboratory diagnosis due to the need for highly qualified personal.

Immunoassays. Enzyme-linked immunosorbent assay (ELISA), based on the use of hyperimmune antisera raised against recombinant NoV virus-like particles (VLPs), have been developed for the detection of NoV antigens in clinical samples but are often highly specific to the immunising VLP (detecting only strains of the same genotype or genetically similar). Progress has been made in the development multivalent antibodies with wide-ranging reactivity allowing the detection of a broad range of HuNoV genotypes and a couple of commercial diagnostic ELISA kits are now available. These commercial ELISA were

submitted to a European multicentre evaluation that concluded that these assays should be used for screening purposes and results should be further confirmed by RT-PCR (Gray *et al.*, 2007).

Genomic amplification by RT-PCR. Nowadays RT-PCR is the most widely used technique for the detection of NoV in human and animal clinical samples (faeces and/or vomits), in food and water samples or even in environmental samples and fomites (Kim *et al.*, 2008; Laverick *et al.*, 2004; Richards *et al.*, 2004; Suffredini *et al.*, 2011; Trujillo *et al.*, 2006). Genomic regions targeted for detection are highly conserved sequences among NoVs in the same genogroup such as the RdRp gene (Ando *et al.*, 1995; Scipioni *et al.*, 2008b; 2008c; Vinje *et al.*, 2003) or more recently a short sequence located at the ORF1-ORF2 junction (Jothikumar *et al.*, 2005; Kageyama *et al.*, 2003; Wolf *et al.*, 2007). Due to the genetic diversity of NoVs one single primer pair will not be able to amplify all existing NoVs. The simultaneous detection of multiple NoV genotypes within different genogroups can be achieved by multiplexing the RT-PCR assays (Shigemoto *et al.*, 2011; Stals *et al.*, 2009; Wolf *et al.*, 2010). Moreover, some NoVs predominantly circulate in humans and animal populations rendering the latter issue less problematic for routine analyses. Real-time RT-PCR (RT-qPCR) has emerged as the gold standard method for the detection of NoVs for the following reasons: (i) a lower detection limit in comparison to conventional RT-PCR and other molecular methods (Beuret, 2004); (ii) less time consuming by the amplification of small PCR products and the absence of post-PCR processing that also limits cross-contamination issues; and (iii) the possibility of quantification by the use of fluorescent probe-based chemistries such as Taqman probes (Mackay *et al.*, 2002; Niesters, 2002). Multiplexing of virus-specific-primers and probes detecting NoVs from different genogroups can offer the advantage of genotyping without sequencing.