Chapter 4: Discussion and perspectives

The importance and the implications of recombination on the evolution of NoV were studied in natural conditions and in experimental conditions throughout this thesis. Three experimental approaches were used and led to following major observations: i) along with point mutations, recombination constitutes a major driven force in NoV evolution and plays a crucial role in the trends of NoV epidemiology in industrialised countries like Belgium; ii) NoV recombination events can be reproduced experimentally without any selection pressure following cellular co-infections by means of the MNV model; iii) crossovers seem to occur according to a similarity-assisted recombination model with the presence of high sequence homology and a stem-loop secondary structure at the recombination hot spot of the NoV genome (e.g. ORF1-ORF2 junction); iv) the exchange of genetic material between NoVs can generate viruses with distinct biological properties from the parental viruses; v) the evaluation of body weight during the course of NoV infection could constitute an alternative of virulence assessment in the absence of clinical signs in wild-type mice and vi) quantitative results obtained by RT-qPCR should always be interpreted with care and their use should be limited to the study of viruses for which no conventional titration methods are available.

The work accomplished in the first part of this thesis enabled gaining insight into the epidemiologic patterns of NoV outbreaks in Western Europe through the monitoring of the NoV strains associated with outbreaks in Belgium between December 2006 and December 2010. Genotyping by conducting phylogenetic analyses of the circulating NoV strains confirmed that NoVs are extremely swift viruses and that this remarkable diversity was not solely due to small-scale nucleotide mutations. Part of the detected NoV strains showed incongruent clustering between partial sequences of the polymerase and the capsid genes among which two hitherto unreported recombinant strains (GII.e/GII.3 and GII.g/GII.1). Throughout the study period, the emergence of novel GII.4 variants and GII recombinants came along with increased NoV activity as observed in 2008 and 2010 suggesting that these strains are fitter from an epidemiological point of view. Our results highlight the importance of recombination in the genetic divergence of NoVs playing a key role in the NoV epidemiology. Thus, the understanding of the underlying mechanisms (e.g. recombination) responsible for the variable phylogenetic dynamics of NoV strains will be of great importance not only for the development of effective and novel strategies for pathogen control but also for understanding the future impact of NoVs.

Thus, the second part of this thesis was dedicated to the experimental study of NoV recombination and more particularly the effects of recombination on the biological properties of the progeny viruses. Because HuNoVs cannot be efficiently cultivated *in vitro*, an experimental model for the *in vitro* generation of MNV recombinants was set up in our laboratory. This study provided the first evidence of intermolecular genetic exchange yielding a viable recombinant virus in an experimental setting for a member of the *Norovirus* genus. Furthermore, this study tended to evaluate if the genetic novelty offered by recombination events could have an immediate impact on the viral behaviour in terms of fitness and virulence of the recombinant virus in comparison to the parental viruses. The experimental model for the generation and detection of recombinant MNVs *in vitro* allowed the isolation of a viable recombinant MNV exhibiting a breakpoint at the ORF1-ORF2 junction previously recognised as a recombination hotspot for recombinants within the *Caliciviridae* family. The crossover was located within a region of perfect identity between the sequences of the 2 parental viruses where a stem-loop secondary structure was described for the anti-genomic RNA sequence. These findings support the model of similarity-assisted recombination in NoVs. The rate of recombination events for MNV in permissive experimental conditions resulting into replication competent genomes was ~ 0.82% and lies within the rates previously reported for other RNA viruses such as picornaviruses (0.13 and 2%). Rec MNV was less fit in cell culture in comparison to the parental MNVs and the smaller plaques and altered growth kinetics exhibited by Rec MNV were probably due to less efficient virus egress. When virulence was evaluated in the natural host, both the parental and Rec MNVs were able to cause systemic infections in immunocompetent mice. Here, we report for the first time significant weight losses consecutive to MNV-1 and WU20 infections at 48 hpi while body weight losses for Rec MNV infected mice were similar to the mock-infected mice. Thus, the evaluation of body weight could constitute a valuable parameter for virulence assessment in the absence of obvious clinical sign in immunocompetent mice.

In conclusion, NoV recombination events enable the generation of new variants with modified phenotypic properties like attenuated virulence in their natural host. Furthermore, the rapid exchange of genetic material through recombination could allow the creation of virus strains with modified cell/tissue/host tropism within a matter of days (Pybus and Rambaut, 2009). Therefore, as previously suggested for other RNA viruses (Graham and Baric, 2010), recombination could be involved in cross-species transmission events and more particularly

zoonotic transmissions. The detection of NoVs in humans and animals raised questions on zoonotic transmissions or the existence of an animal reservoir that yet still needs to be elucidated. Until today all sequences obtained from human clinical samples clustered with human NoV sequences and most NoVs detected in animals, although related to HuNoVs, clustered into genotypes or genogroups specific to each species. Even if the majority of the data available today tends to exclude interspecies transmission, several findings are in favour of its occurrence. The latter will be presented according to the four steps involved with the emergence of host-switching viruses: exposure, infection, spread and adaptation (Parrish *et al.*, 2008).

Exposure. NoVs were detected in domestic and wild animals in close contact with humans either kept for subsistence (cattle, pigs, sheep), ornamental (lion cub), research (mice) or companionship (dogs) purposes. Persons at particular risk would be not only animal owners (e. g. dog owners and farmers) but also people who are in contact due to professional activities (e. g. veterinarians, butchers, scientists, zookeepers and slaughters). Although no animal NoVs have been detected in humans, Widdowson and collaborators suggested IgG reactivity to BoNVs to be more common in veterinarians than in the control population (Widdowson *et al.*, 2005). Another study showed that work tending to livestock and the presence of a dog in the close surrounding of the house were associated with increased odds for a seroresponse to NoVs (Peasey *et al.*, 2004). These results indicate that some populations might be at risk and be more susceptible to be infected by animal NoVs. NoVs are oro-faecally transmitted and very resistant in environmental conditions. Consequently, apart from direct contact, indirect transmission can occur through the ingestion of contaminated recreational or drinking waters (infecting both animals and humans) or the consumption of contaminated bivalve shellfish. Indeed shellfish are capable of accumulating both human and animal NVs (Costantini *et al.*, 2006; Zakhour *et al.*, 2010) even though specific binding of animal NoVs to oyster tissues was found to be less efficient. Whether NoVs can be transmitted by produce of animal origin remains unclear. Human-like NV sequences were detected in retail pork meat but the source of contamination remained unclear and could originate from food handling (Mattison *et al.*, 2007).

Infection and spread. No animal NoVs have been detected in human clinical samples until date and it is unclear whether animal NoVs can infect humans. Serological studies showed higher antibody titres against recombinant capsid proteins of BoNVs in veterinarians than in the control group suggesting infection by BoNVs in this risk population (Widdowson *et al.*,

2005). Contradictive results were obtained for cross-reactivities between HuNoV and BoNoV epitopes (Batten *et al.*, 2006; Han *et al.*, 2005; Oliver *et al.*, 2006; Widdowson *et al.*, 2005), as a consequence this phenomena cannot be ruled out. Indeed, the higher prevalence of IgA against NoV found in persons with dogs in their near environment or with regular interactions with livestock could be explained by the existence of cross-reactive epitopes between human and animal NoVs. Provided that results were interpreted with care, these data would suggest the possibility of animal NoVs to infect humans. Carbohydrate antigens of the histo-blood group family, known to be shared among various mammal species, have been shown to act as receptor for different caliciviruses including NoVs (Ruvoen-Clouet *et al.*, 2000; Shirato-Horikoshi *et al.*, 2007; Tan and Jiang, 2005) .This characteristic could enhance interspecies, including zoonotic, NoV transmissions. Conclusions from virus-host interaction studies evaluate chances of BoNoVs to infect humans to be thin seeing that the alphaGal epitope involved with BoNoV VLP binding is not expressed in tissues of human or porcine origin (Mauroy *et al.*, 2011; Zakhour *et al.*, 2009). Similarly, porcine GII.11 epitopes failed to fix on human tissues and saliva whereas fixation was successful on stomacal and intestinal tissues of porcine and bovine origin (Farkas *et al.*, 2005) (Zakhour *et al.*, 2009).

On the other way round, HuNoV-like sequences have been detected in porcine and bovine faecal samples (Mattison *et al.*, 2007; Nakamura *et al.*, 2010; van Der Poel *et al.*, 2000) but whether HuNoVs replicated in the animals remains unclear. Under experimental conditions, human GII NoVs successfully infected gnotobiotic young pigs and calves and infections were accompanied by mild clinical signs limited to the intestinal tract (Cheetham *et al.*, 2006; Souza *et al.*, 2007; 2008; Takanashi *et al.*, 2011). These results are in favour of the possibility of HuNoV to sporadically infect these animals. To comfort this hypothesis, healthy cows' colostrums were shown to contain antibodies able to react to HuNoV VLPs (Murakami *et al.*, 2010). Furthermore, serological evidence of GIV NoV infections were found in cats and dogs in Italy, again cross-reactions with other NoV strains could not be ruled out (Di Martino *et al.*, 2010).

Adaptation. Even if sporadic cross-species transmissions are common, a number of specific changes are required for the virus to infect, replicate and transmit efficiently in its novel host species. The probability of a virus to switch host species will depend upon different factors including i) virus potential, ii) host susceptibility or iii) the chance occurrences. In this part we will develop more specifically the ability of viruses to successfully adapt to a new host. From an evolutionary point of view, viral variability is thought to enhance the probability of viruses

to adapt to a new host (Parrish *et al.*, 2008). In this context, RNA viruses, and especially positive RNA viruses, due to a high number of nucleotide substitutions, could be more inclined to transgress host barriers. These high mutation rates are imputable to i) the error prone replication of RNA viruses with a lack of proofreading activity of the polymerase, ii) the rapid virus replication with short virus generation times and iii) the creation of large virus populations. NoVs do not constitute an exception and high evolutionary rates of 10^{-2} to 10^{-3} nucleotide substitution/site/year were found (Siebenga *et al.*, 2010b; Victoria *et al.*, 2009). On top of small-scale mutations, recombination events have been largely implicated in the generation of viral diversity. Genetic recombination could enable the creation of new combinations of genetic materials, generating more dramatic genomic changes than point mutations. Phylogenetic analyses of NoV genomes have shown that recombination is likely to occur both in human and animal NoVs (Bull *et al.*, 2005; Martella *et al.*, 2009; Oliver *et al.*, 2004; Thackray *et al.*, 2007). Furthermore, experimental evidence of NoV recombination was provided by an *in vitro* co-inoculation study (Mathijs *et al.*, 2010). This study showed that a NoV recombination event could yield a recombinant virus exhibiting biological properties that differ from the parental ones as assessed *in vitro* and *in vivo*. Similarly, reassortment seems to play a crucial role in host switching for influenza A viruses (Parrish and Kawaoka, 2005). Thus, the fact that NoVs can undergo genomic reshuffle through recombination could enhance their adaptation to a new host after cross-species transmissions. Figure 18 shows the different probable co-infection scenarios between human and porcine NoVs that could lead to the generation of interspecies recombinant NoVs with altered biological properties. This scenario was preferred upon others because i) PoNoV are genetically more related to HuNoV both clustering into GII, ii) the experimental evidence of porcine susceptibility to HuNoV infections and iii) the detection of HuNoV-like sequences in porcine faeces and detail meat. Co-infections in either pigs or humans by direct contact or through the ingestion of contaminated matrices (e.g. shellfish for humans) could give opportunities for recombination to occur between related GII NoVs. Here, we have chosen the porcine species but a similar scenario could be described for GIV NoV between humans and dogs. Moreover, recent evidence of potential GIV interspecies recombination has been suggested by Martella and collaborators (Martella *et al.*, 2009).

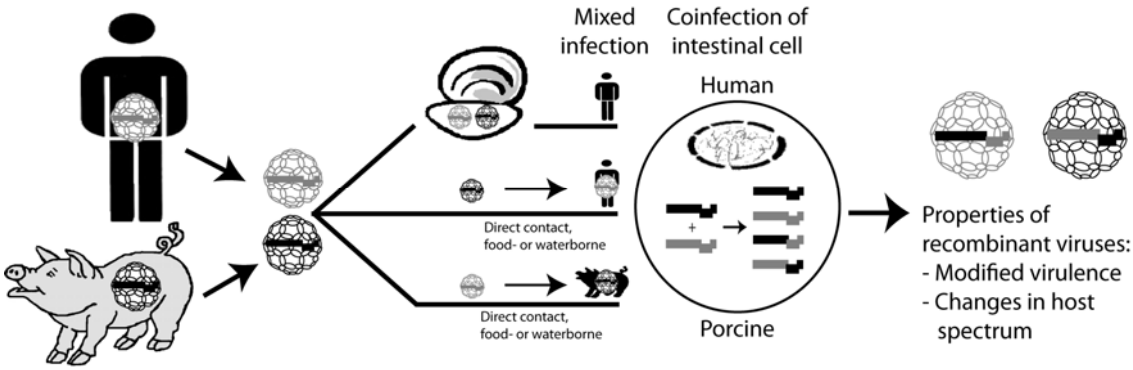

Figure 18: Schematic view of different co-infection scenarios between porcine and human noroviruses leading to recombination and the emergence of recombinants with new properties.

As NoVs are constantly evolving viruses either by the accumulation of point mutations or through recombination, the latter could more particularly play a role in the enhancement of inter-species transmission including zoonotic infections with unpredictable outcomes. Thus, the monitoring of NoVs circulating both in humans and animals will remain essential when it comes to the evaluation of the role of animals in the NoV epidemiology.

The work conducted in this thesis confirmed that recombinant NoVs constitute a non-negligible part of the circulating NoVs in human populations in our counties suggesting its major importance in the NoV epidemiology and evolution. Thus, molecular epidemiological studies on NoVs should include genetic amplifications of both the polymerase and the capsid gene regions as most recombination events occur at the ORF1-ORF2 junction. Furthermore, a common language still needs to be adopted in order to compare data between the different research teams and the standardised nomenclature for NoVs developed by the Noronet consortium will therefore constitute an important breakthrough. The monitoring of the circulating NoV strains should be carried on in the future and the results should be compared with international data in order to confirm and understand the success of the recombinant NoV strains and more particularly those possessing GII.e and GII.g polymerase genes. Additionally, due to the high genetic diversity of NoVs, a continuously updated overview of the strains predominantly and contemporaneously involved in large and cost-effective gastro-enteritis outbreaks will be needed to target the development of vaccines, therapeutic strategies and/or preventive measure on these particular strains.

When reproduced *in vitro*, a similarity-assisted NoV recombination event was shown to enable the creation of a novel variant with altered fitness in cell culture and attenuated virulence in its natural host. Unfortunately, a single viable recombinant virus could be isolated after multiple attempts implicating different experimental settings. Thus, the MNV model appears to be suitable for the study of NoV recombination in cell culture in the absence of available culture systems for HuNoVs although various adaptations could be made in order to enhance the *in vitro* recombination frequency (e.g. higher sequence similarity between parental genomes, the application of selection pressure and the use of another cell type). The altered phenotypic features of Rec MNV *in vitro* were confirmed *in vivo* but only few significant differences were found in virus loads in organ tissues estimated by plaque assay. In order to interpret the results, the influence of various points in the experimental set-up, such as i) increasing the size of the mouse groups, ii) using immunodeficient mice for infection, iii) lowering the dose of inoculation and iv) limiting the number of cell passages before infection, should be evaluated. Interestingly, body weight losses at 48 hpi differed significantly between the parental and Rec MNVs indicating that this parameter could be used for virulence assessment in the absence of obvious clinical signs in wild-type mice.

On the whole, MNV recombination resulted in the generation of a novel genetic combination that affected the biological properties of the new virus. The use of reverse genetics could be of particular interest for the creation a panel of genetic rearrangements that could be evaluated *in vitro* and *in vivo* and allowing a more accurate understanding of the implications of recombination on the biological properties of NoVs. The knowledge of such consequences could directly allow a better estimation of the NoV epidemiology in the future.

This thesis also allowed the implementation of an *in vitro* and *in vivo* NoV study model in our laboratory. Future studies, including the evaluation of biocide efficiencies against NoVs, will be realised using this model.

Chapter 5: Summary – Résumé

Summary

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. In Belgium, NoVs have been identified as the first cause of foodborne gastroenteritis outbreaks before *Salmonella* spp and *Bacillus cereus*. Transmission routes are multiple and outcomes of NoV infections can be particularly severe in health care settings due to the presence of vulnerable patients with underlying severe illnesses. NoVs have been detected in a broad variety of animal species (Scipioni *et al.*, 2008a), raising important, yet partly unanswered, questions about the possibility of zoonotic transmissions and the existence of an animal reservoir for NoVs.

NoVs are highly swift viruses and characterised by great genetic variability that can be attributed to two main evolutionary forces: point mutation and recombination. Both mechanisms allow NoVs to continuously generate mutant genomes. The study on NoV biology including recombination has long been hampered by the lack of cell culture systems and small animal models. Consequently, the discovery of cultivable NoVs that naturally infect laboratory mice gave new perspectives in NoV research. Despite the description of numerous human and animal recombinant NoVs by phylogenetic analysis (Bull *et al.*, 2007), no experimental evidence of NoV recombination after cell culture co-infection is available yet.

The aim of this thesis was to evaluate the importance and the potential implications of NoV recombination on the evolution of NoV in natural conditions and in experimental conditions.

NoVs found associated with foodborne suspected gastroenteritis outbreaks reported to the Belgian Scientific Institute of Public Health between December 2006 and December 2010 were systematically characterised. Therefore, we established a genotyping procedure based upon phylogenetic analyses of the partial sequences of the polymerase and the capsid genes.

A second part was dedicated to the *in vitro* creation of NoV recombinants after the set up of the murine model. Molecular methods allowing the distinction between two parental wild-type MNVs in different regions of the genomes were implemented in order to detect hybrid genomes among the progeny viruses of co-infections. Furthermore, the consequences of recombination on the biological properties of the recombinant viruses in comparison with the parental ones were assessed *in vitro* and *in vivo.*

Among all foodborne suspected outbreaks, NoVs were implicated in 11.8% of the cases and responsible for 34.5% of the persons reported ill. Genogroup 2 (GII) NoVs predominated widely (90.4% of all outbreaks) and GII genotype 4 (GII.4) was detected in 16 of the 28 (57.1%) typed outbreaks. GII.4 2006 variants were repeatedly detected circulating with variants 2007 and 2008 until 2009 before being replaced by a novel variant GII.4 2010 the year after. The latter variant constituted the only GII.4 variant circulating in 2010 and was involved in 8/18 typed outbreaks reported in the winter 2009-2010. Phylogenetic analyses allowed the identification of 5 different combinations of NoVs recombinants associated with 14 outbreaks; four novel GII intergenotype and intersub-genotype recombinants (GII.e/GII.3, GII.g/GII.1, GII.4 2006b/GII.4 2007 and GII.4 2010/GII.4 2010b) were detected along with 1 previously published recombinant (GII.e/GII.4 2007). For all the recombinants, the recombination cross-over was located at the junction of the genes that code for the polymerase and the capsid proteins.

Using the murine NoV (MNV) model, we investigated recombination between two wild-type MNV strains co-infecting mouse monocyte and macrophage cell line (RAW 264.7). A PCR-based genotyping tool capable of discriminating between parental viruses allowed the detection of a viable chimeric virus (Rec MNV) among the progeny viruses. Genetic analysis confirmed the Rec MNV genome to be a chimera created by a homologous recombination event located at the overlap between the ORFs coding for the polymerase and the capsid proteins. In comparison with the parental viruses, Rec MNV showed distinct multiplication kinetics and produced significantly smaller plaques in cell culture. After per oral infections of immunocompetent Balb/c mice with 5.10^6 plaque forming units of parental viruses and Rec MNV, Rec MNV infected mice showed lower body weight losses at days 2 and 3 post-infection (2-3 dpi) compared to those infected with the parental viruses. Rec MNV was more rapidly cleared from faeces because, contrary to what was observed for the parental viruses, no detectable Rec MNV viruses were found at 3 dpi. Meanwhile, virus titres observed for Rec MNV in faeces and in different organs (small intestine, spleen, mesenteric lymph nodes, left lung) at 2-3 dpi did not differ much from those observed for the parental viruses. Also, the presence of detectable levels of viruses in all organs analysed suggest that, similarly to the parental viruses, Rec MNV can induce a systemic infection and disseminate beyond organs associated with the digestive tract.

The study of NoVs implicated with suspected foodborne gastroenteritis outbreaks in Belgium over a 4-year period highlight the importance of NoVs for public health in our regions. NoVs

circulating during this period exhibit great genetic variability due to point mutations and recombination events. The GII.4 2006 until then predominantly present across the globe was successfully displaced by the newly emerging GII.4 2010 variant. Similarly, GII.b recombinants previously considered as endemic on the European continent seem to have been substituted by the nascent recombinants GII.e in 2009 and GII.g in 2010. Among these recombinants, two novel recombinant combinations (GII.e/GII.3 and GII.g/GII.1) have been characterised. Our results also suggested that part of the viruses of the highly successful GII.4 lineage were in fact mosaics of former GII.4 NoVs suggesting recombination might play a role in the divergent evolution of GII.4 NoVs. The emergence of new GII.4 variants together with GII recombinants could explain the explosive increase in reported cases of gastroenteritis that was observed in 2008 and 2010.

The high genetic diversity of NoVs plays a key role in the difficulty to implement efficient prophylactic and therapeutic measures. Consequently, the determination of NoVs predominantly involved in large and cost-effective gastroenteritis outbreaks will allow targeting the development of these procedures on the most prevalent strains. Moreover, taking into account that NoVs undergo point mutations and recombination, the availability of regularly updated vaccines will require the assessment of contemporaneously circulating strains.

Through the use of the MNV model, the present work offers the first experimental evidence of NoV recombination after the co-inoculation in cell culture of two distinguishable MNV strains. The obtaining of a recombinant in permissive conditions without any pressure of selection suggests that the phenomenon is not rare. Nevertheless, different adaptations could be foreseen in order to use the model for the study of NoV recombination. Similarly to what was observed for recombinant HuNoVs in natural conditions, the localisation of the crossing-over at the ORF1-2 overlap with the identification of a stem-loop structure at the anti-genomic strand is in line with the recombination model proposed by Bull and collaborators (2007). This model proposes that the exchange of genetic material occurs during the switch of the polymerase from one genomic strand to the other during RNA negative strand transcription into positive strand RNA.

The evaluation of the biological characteristics of Rec MNV in comparison to the parental viruses both *in vitro* and *in vivo* suggests that recombination can result in the creation of chimeric viruses whose properties differ from the parental ones. Its consequences could have a major impact on NoVs evolution like the creation of highly pathogenic viruses or viruses with broadened host ranges. Indeed, the adaptation of a virus in a novel host by point

mutations could take years whereas recombination can quickly generate the required genetic combinations.

This thesis allowed the implementation of an *in vitro* and *in vivo* NoV study model in our laboratory. The results obtained both in natural conditions for HuNoVs and in experimental conditions for MNVs indicate that recombination, along with point mutation, seem to be highly implicated in the evolution of NoVs. Thus, the understanding of these phenomena will be crucial if therapeutic and prophylactic measures are to be implemented for NoV infections.

Résumé

Les norovirus (NoVs) constituent une cause majeure de cas sporadiques et des épidémies de gastro-entérites chez les humains de tout âge. Ils sont responsables d'environ 90 % des épidémies de gastro-entérites non bactériennes dans les pays industrialisés. En Belgique, les NoVs représentent la première cause de gastro-entérite d'origine alimentaire avant *Salmonella* spp et *Bacillus cereus*. Les voies de transmission peuvent être multiples et les infections à NoV peuvent avoir des impacts majeurs sur la santé publique particulièrement dans les établissements de soin avec la présence de personnes plus vulnérables souffrant d'autres pathologies. Des NoVs génétiquement proches des NoVs humains ont été identifiés chez des animaux domestiques tels que porcs, veaux et chiens (Scipioni *et al.*, 2008a). Par consequent, la détection des NoVs chez les animaux pose la question de l'existence d'une transmission zoonotique ou de réservoir animal.

Les NoVs sont des virus très dynamiques et sont caractérisés par une très grande variabilité génétique expliquée par deux mécanismes majeurs : les mutations ponctuelles et la recombinaison. Ces forces évolutives offrent aux NoVs l'opportunité de créer continuellement de nouveaux virus. La connaissance de la biologie des NoVs et notamment de la recombinaison reste limitée par le manque de systèmes de culture et de modèles animaux. Ainsi, la découverte de NoVs infectant naturellement la souris a permis d'entrevoir de nouvelles perspectives dans la recherche sur les NoVs. Malgré la description d'un grand nombre des NoVs recombinants humains et animaux par des analyses phylogénétiques (Bull *et al.*, 2007), il n'y a aucune donnée expérimentale de recombinaison après co-infection en culture cellulaire par des NoVs.

Le but de ce travail était l'évaluation de l'importance et des conséquences de la recombinaison sur l'évolution des NoVs en conditions naturelles pour les NoVs humains et en conditions expérimentales dans le modèle murin.

Les NoVs associés à des épisodes de gastro-entérites suspectées d'origine alimentaire rapportés à l'Institut Scientifique de Santé Publique entre décembre 2006 et décembre 2010 ont été analysés et caractérisés de façon systématique. Pour cela, nous avons établi une procédure de génotypage basé sur l'obtention de séquences partielles des gènes de la polymérase et de la capside suivie d'analyses phylogénétiques.

Une deuxième partie était consacrée à la génération *in vitro* de NoV recombinants, après la mise au point d'un modèle murin. Des méthodes moléculaires permettant la différenciation

entre deux virus parentaux ont été mises au point pour différentes régions du génome pour la mise en évidence de génomes hybrides parmi les virus issus de co-infections. De plus, les conséquences de la recombinaison ont été évaluées en comparant les propriétés biologiques du virus recombinant avec les virus parentaux *in vitro* et *in vivo*.

Parmi toutes les épidémies suspectées d'origine alimentaire, les NoVs étaient impliqués dans 11,8 % des cas et responsables de 34,5 % des personnes malades. Les NoVs de génogroupe 2 (GII) prédominaient de façon importante (90,4 % de tous les épisodes) avec le GII génotype 4 (GII.4) détecté dans 16 des 28 épisodes typés (57,1 %). Les variants GII.4 2006b ont été identifiés à plusieurs reprises en co-circulation avec les variants 2007 et 2008 jusqu'en 2009 avant d'être remplacés par un nouveau variant GII.4 2010 l'année suivante. Ce même variant était l'unique génotype GII.4 circulant en 2010 et était impliqué dans 8 des 18 épisodes typés durant l'hiver 2009-2010. Les analyses phylogénétiques ont mis en évidence 5 types de NoVs recombinants impliqués dans 14 épisodes dont 4 nouveaux recombinants GII inter génotype et inter sub-génotype (GII.e/GII.3, GII.g/GII.1, GII.4 2006b/GII.4 2007 and GII.4 2010/GII.4 2010b) et un recombinant décrit précédemment (GII.e/GII.4 2007). Pour tous ces NoVs recombinants, le point de recombinaison était toujours situé à la jonction entre les gènes codant les protéines de la polymérase et de la capside.
En utilisant le modèle du NoV murin (MNV), nous avons investigué la recombinaison entre deux souches sauvages de MNV co-infectant des cellules de la lignée hématopoïétique de la souris. Un outil de génotypage basé sur la PCR a permis la discrimination des virus parentaux autorisant la détection d'un virus chimérique viable (Rec MNV) parmi les virus de nouvelle génération. D'après l'analyse génétique, le génome du virus recombinant était une chimère issue d'un évènement de recombinaison homologue localisé à l'endroit de chevauchement des cadres de lecture ouverts codant les protéines de la polymérase et de la capside. En comparaison avec les virus parentaux, le Rec MNV avait des courbes de multiplication distinctes et produisait des plages de lyse significativement plus petites en culture de cellules. Après infection orale de souris Balb/c immunocompétentes avec 5.10^6 unités formant plages des virus parentaux et de Rec MNV, les souris infectées avec le Rec MNV avaient des pertes de poids moindres que celles infectées avec les virus parentaux à 2 et 3 jours après infection (2-3 dpi). Toutefois, les charges virales détectées dans les matières fécales et différents organes (intestin grêle, rate, ganglions mésentériques, poumon gauche) à 2 et 3 dpi pour Rec MNV ne différaient que peu par rapport à celles observées pour les virus parentaux. Aussi, les

titres observés pour le Rec MNV suggéraient une capacité similaire aux souches parentales à induire une infection généralisée et à disséminer au-delà des organes associés au tube digestif.

L'étude des NoVs impliqués dans les épisodes de gastro-entérites suspectées d'être d'origine alimentaire en Belgique entre décembre 2006 et décembre 2010 soulève l'importance de ce virus dans la santé publique humaine dans nos contrées. Les NoVs circulant durant cette période présentent une diversité génétique remarquable soit par des mutations ponctuelles ou grâce à des évènements de recombinaison. Le variant GII.4 2006, jusqu'à lors prédominant à travers le monde, a été remplacé avec succès par un variant nouvellement émergent GII.4 2010. De la même façon, les recombinants GII.b précédemment décrits comme étant endémique en Europe de l'Ouest semblent être remplacés par les recombinants GII.e en 2009 et GII.g en 2010. Parmi ces recombinants, deux types de recombinants auparavant inconnus (GII.e/GII.3 et GII.g/GII.1) ont été caractérisés. De plus, l'étude suggérait que, parmi les GII.4, certains seraient en fait des mosaïques de GII.4 ayant circulé auparavant et indiquait que la recombinaison pourrait contribuer à l'évolution divergente à la base du succès des NoVs GII.4. L'apparition de nouveaux sous-types GII.4 ainsi que de nouvelles souches GII recombinantes pourrait expliquer l'augmentation explosive des cas de gastro-entérites observée en 2008 puis en 2010. L'extrême diversité génétique des NoVs joue un rôle majeur dans la difficulté d'élaboration de traitements et d'outils prophylactiques tels que les vaccins. Par conséquent, la caractérisation des NoVs qui sont préférentiellement impliqués dans de larges et coûteux épisodes de gastro-entérite pourrait permettre de cibler la mise au point de ces procédures sur ces souches en particulier. De plus, compte tenu de l'évolution rapide des NoVs, un suivi continu des souches de NoV qui circulent de façon contemporaine dans la population humaine est indispensable afin d'adapter régulièrement les méthodes de diagnostic.

Grâce au modèle du MNV, ce travail offre la première évidence expérimentale de la recombinaison chez les NoVs après la co-inoculation en culture cellulaire de deux souches de MNV distinguables. L'obtention d'un virus recombinant en conditions permissives et sans qu'aucune pression de sélection n'ait été appliquée suggère que le phénomène n'est pas rare. Cependant, différentes adaptations du système pourraient être envisagées afin qu'il puisse servir de modèle d'étude pour la recombinaison chez les NoVs. De façon similaire à ce qui a été observé pour les NoVs humains recombinants en conditions naturelles (Bull *et al.*, 2005), la localisation du point de recombinaison à la jonction des ORF1-2 où une structure secondaire de type tige-boucle a été identifiée au niveau du brin anti-sense du génome rejoint

le modèle de recombinaison proposé par Bull et collaborateurs (Bull *et al.*, 2007). Ce modèle propose que l'échange de matériel génétique s'effectue lors du saut de la polymérase d'un brin génomique à l'autre lors la transcription du brin d'ARN négatif en ARN positif. L'évaluation *in vitro* et *in vivo* des caractéristiques biologiques du Rec MNV par rapport aux souches parentales suggère que la recombinaison peut aboutir à la création de virus chimériques dont les propriétés diffèrent des virus dont ils sont issus. Par conséquent, la recombinaison pourrait avoir des répercussions majeures sur l'évolution des NoVs tels que des changements de tropisme d'hôte. En effet, après le franchissement d'une barrière d'espèce, l'adaptation complète du virus à un nouvel hôte par des mutations ponctuelles peut nécessiter des années alors que la rapidité avec laquelle la recombinaison permet de créer de nouvelles combinaisons génétiques pourraient favoriser ce phénomène. Les résultats obtenus tant en conditions naturelles qu'expérimentales indiquent que la recombinaison constitue, au côté des mutations ponctuelles, une force majeure d'évolution des NoVs.

Le travail présenté dans cette thèse a permis la mise au point d'un modèle d'étude des NoV *in vitro* et *in vivo* dans notre laboratoire. Les résultats obtenus dans des conditions naturelles et expérimentales pour les NoVs humains et les NoVs murins respectivement indiquent que la recombinaison joue un rôle majeur dans leur évolution. Il sera impératif de tenir compte de ce phénomène si un vaccin est mis au point pour lutter contre les infections à NoV.