Norovirus recombinants: recurrent in the field, recalcitrant in the lab – a scoping review of recombination and recombinant types of noroviruses

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Abstract

Noroviruses are recognized as the major global cause of sporadic and epidemic non-bacterial gastroenteritis in humans. Molecular mechanisms driving norovirus evolution are the accumulation of point mutations and recombination. Intragenotypic recombination has long been postulated to be a driving force of GII.4 noroviruses, the predominant genotype circulating in humans for over two decades. Increasingly, emergence and re-emergence of different intragenotype recombinants have been reported. The number and types of norovirus recombinants remained undefined until the 2007 Journal of General Virology research article ‘Norovirus recombination’ reported an assembly of 20 hitherto unclassified intergenotypic norovirus recombinant types. In the intervening decade, a host of novel recombinants has been analysed. New recombination breakpoints have been described, in vitro and in vivo studies supplement in silico analyses, and advances have been made in analysing factors driving norovirus recombination. This work presents a timely overview of these data and focuses on important aspects of norovirus recombination and its role in norovirus molecular evolution. An overview of intergenogroup, intergenotype, intragenotype and ‘obligatory’ norovirus recombinants as detected via in silico methods in the field is provided, enlarging the scope of intergenotypic recombinant types to 80 in total, and notably including three intergenogroup recombinants. A recap of advances made studying norovirus recombination in the laboratory is given. Putative drivers and constraints of norovirus recombination are discussed and the potential link between recombination and norovirus zoonosis risk is examined.

INTRODUCTION

Noroviruses (NoVs) belong to the Caliciviridae family of small, non-enveloped, positive sense, single-stranded RNA viruses, currently divided into the five approved genera Vesivirus, Lagovirus, Neboivirus, Sapovirus and Norovirus, while additional genera have been proposed [1]. Detected in a wide range of mammalian species, NoVs cause gastroenteritis of varying severity in their animal hosts [2]. Human noroviruses (HuNoVs) are recognized as the major global cause of sporadic and epidemic non-bacterial gastroenteritis [3, 4], with significant morbidity and mortality in impoverished developing countries [5, 6] and a high economic impact in developed countries [7]. Despite their significance, no viable cell culture system existed for the study of HuNoVs until the recent report of low-level infection of cultured human B cells [8] and the advent of the human enteroid system [9]. While practicability of these new cell culture systems still presents hurdles, the murine norovirus (MuNoV), replicating efficiently in murine dendritic or macrophagic cells [10, 11], currently remains the model of choice for in vitro study of NoVs and in vivo infection of a genetically tractable host.

HuNoVs and MuNoVs share many similarities in terms of their genome structure. The HuNoV linear, single-stranded, polyadenylated positive-sense, ca. 7.5 kb long RNA genome is classically divided into three ORFs, with a fourth described for MuNoVs [12]. The 5’ proximal ORF1 encodes a large polyprotein that is co- and post-translationally cleaved by protease-catalysed mechanisms into the six non-structural viral proteins (NS1/2 to NS7) [6]. ORF2 and ORF3 encode the structural components of the virion, major and minor capsid protein, VP1 and VP2, respectively. VP1 itself consists of a conserved shell (S) and two protruding (P) domains, of which the conserved P1 enhances particle stability, while the exposed, variable P2 forms binding clefts for virus receptors and harbours antigenic epitopes at
the capsid exterior. ORF4, entirely overlapping the 5' end of ORF2, encodes an antagonist of innate immunity, virulence factor VF1 [6].

NoVs are genetically classified into six established genogroups (GI–GVI), while a seventh (GVII) was recently proposed [13]. Genogroups are further divided into at least 38 genotypes [14, 15] and GII.4 strains are additionally sub-typed into variants. Since the mid-1990s, classification into genogroups and genotypes has been based on amino acid sequence analysis of the complete VP1 capsid protein, with an updated cut-off threshold of a minimum of 15% pairwise difference proposed for classification of a new genotype. To account for the common occurrence of recombination in the ORF1–ORF2 overlapping region, a dual-nomenclature system based on complete capsid sequences and the RNA polymerase region in ORF1 [14] was recently established by consensus of the International Norovirus Working Group. The remarkable level of variability within the NoV genus reflects the high level of continuous viral evolution therein.

Molecular mechanisms driving NoV evolution are the accumulation of point mutations (or genetic drift) and recombination. In any given virus, the accumulation of point mutations generally leads gradually to genetic variation and the generation of viral quasispecies [16]. Recombination, the complex molecular process by which a fragment of DNA is reciprocally exchanged between homologous chromosomes, serves as the basis of evolution/sexual reproduction in eukaryotes [17] and, in fact, deeply impacts the evolution of all biological entities. In prokaryotes and viruses, recombination (or lateral gene transfer or gene conversion) involves non-reciprocal replacement or addition of genome sequences rather than exchange [17, 18] and can create considerable changes in a given viral genome, allowing for antigenic shifts [19], changes in receptor or host tropism [20], and pathogenesis and fitness modifications to shape viral epidemiology [21].

Intragene recombination has long been postulated to be a driving force of GII.4 NoVs, which have been the predominant genotype circulating in humans for over two decades [22–24]. This position has only recently been challenged by emergence and re-emergence of different intergenotype recombinants modifying long-term global NoV genetic diversity trends [25–31]. Recombination is clearly a widespread evolutionary mechanism used by NoVs.

Here, we review the different aspects of NoV recombination and its role in NoV molecular evolution. We give a comprehensive overview of intergenogroup, intergenotype and intragenotype (and their importance in the GII.4 lineage) and obligatory NoV recombinants, as detected via \textit{in silico} methods in the field. We then provide a recap of advances made studying NoV recombination in the lab. Putative drivers and constraints of NoV recombination are discussed and the potential link between recombination and norovirus zoonosis risk is briefly examined.

**MECHANISMS OF NOV RECOMBINATION – THE ORF1/2 OVERLAPPING HOTSPOT AND LESS COMMON BREAKPOINTS**

Typical as well as atypical recombination breakpoints have been described along the length of the NoV genome. While predictive recombination tools and similarity plots between putative recombinant genomes and suspected parental genomes have suggested recombination at breakpoints within ORF2 in several genomes of MuNoV field strains [32], sequence analysis of field HuNoV strains has overwhelmingly shown the predominant recombination breakpoint to lie in the highly conserved ORF1/ORF2 overlap corresponding to the junction of RdRp and capsid sequences [33]. The region is considered as a negative-strand subgenomic RNA promoter site, leading to the development of a model for NoV recombination, which combines the copy-choice model of recombination in which recombinant RNA molecules are generated via template switch of the RdRp, with an internal initiation mechanism for subgenomic synthesis [33, 34]. Both the standardized NoV nomenclature (as described above) and current genotyping assays are designed to accommodate the ORF1/2 recombination hotspot [14]. Thus, e.g. the Norovirus Automated Genotyping Tool (NoroNet) [35] assigns NoV sequences to a NoV genogroup, and offers unequivocal information on RdRp and capsid-affiliation on either side of the ORF1/2 overlap based on genetic homology and phylogenetic inferences.

However, atypical recombination breakpoints have been observed, amending the paradigm of ORF1/2 recombination. As such, an atypical recombination event located at the 3’ end of the NoV polymerase gene (at nt position 4889) was first described in the GII.4 recombinant Hu/771/2005/IRL (GenBank accession number EF219487) [36]. Recently, similar atypical breakpoints were observed in epidemic GII.4 variants at nt position 4,834 of the GII.4 US95/96/GII.4 Kaiso_2003 recombinant strain Hu/GII.P4/VIG246/2003/BRA (GenBank accession numbers KU756290–KU756293) and at nt position 5,002 of the strain Hu/GII.P4/2A1049/2009/BRA GII.4 Den_Haag-2006b/GII.4 Yerseke_2006a (GenBank accession numbers: KU756294 and KU756295) [37]. Interestingly, there seems a marked tendency for GIL.P7/GIL.6 viruses to also harbour breakpoints located near the 3’ end (C terminus) of the RdRp and at least 40 nt upstream of the overlapping region of ORF1 and 2. The breakpoint in 105 of 112 analysed GIL.P7/GIL.6 sequences was located at nt position 5009 in reference strain GII/Hu/China/2009/GII.P7-GII.6/Beijing (GenBank accession number KX752057) in the absence of a known RNA promoter at this site [38]. An additional ORF2/3 junction breakpoint was reported for GII/4 variants [39–41] and sequences of GII.4 2008 variant viruses have been reported to consistently exhibit a 300–500 bp long mosaic fragment in the ORF2 P2 domain in addition to the typical ORF1/2 breakpoint [42]. In a recent study, only in one of 21 GIL.2 recombinant strains was the recombination breakpoint located within the ORF1/2 overlapping region, while in 15
strains it was located within ORF1 and in five strains within ORF2 [43]. Low frequencies of recombination in the VPg, protease and 3' end of the RdRp coding region as well as the VP1 S domain of MuNoVs were reported by Zhang et al. [44]. Such a recombination in the absence of an obvious RNA promoter or triggering secondary structure has been tentatively purported to suggest that at atypical recombination sites, recombination may have arisen by other mechanisms to those that induce a breakpoint in or around the ORF1/2 overlap [34]. The possibility of non-replicative recombination by which randomly cleaved RNA strands are self-ligated or joined by cellular enzymes has been demonstrated for other positive-sense single-stranded RNA viruses [45–48], and may be considered in this context. Proposed models for replicative and putative non-replicative NoV recombination, as adapted from Bull et al. [34] and a general model for RNA virus recombination [48], respectively, are shown in Fig. 1. Studies in other RNA viruses have shown that observed recombination breakpoints probably only represent a subset of those that are actually generated, and are the ones that are maintained in the viral population after a rigorous functional selection [45, 49]. The same probably holds true for the typical ORF1/2 NoV breakpoint as well as less frequent other breakpoints distributed across the virus genome (discussed further below).

**NOROVIRUS RECOMBINATION IN THE FIELD**

**Intergenogroup and intergenotype recombinants**

Since the first description of a naturally occurring HuNoV recombinant in 1997 [50], recombinant NoVs have been reported worldwide. The actual number and types of recombinants remained undefined until 2007, when Bull et al. published an assembly of 20 hitherto unclassified intergenotypic NoV recombinant types. The authors confirmed seven NoV GI recombinants collectively belonging to the recombinant genotype GI.2/GI.6, 17 prototype GII recombinants, all of which were a combination of one of eight different polymerase genotypes and one of nine different capsid genotypes, and three GIII recombinants.
categorized into two recombinant types NoV GIII.1/GIII.2 and NoV GII.2/GII.1 [34].

In continuation of this list, Table 1 of the present review comprehensively compiles first reports (to the knowledge of the authors) of novel intergenogroup and intergenotype recombinants reported over the intervening ten years. The recombinant type list (Table 1), was assembled following an exhaustive Medline (accessed via PubMed) (www.ncbi.nlm.nih.gov/pubmed) and GenBank (www.ncbi.nlm.nih.gov/genbank/) search, including literature and database hits published from July 2007 to February 2018. PubMed search terms were as follows: (‘norovirus’[MeSH Terms] OR ‘norovirus’[All Fields]) AND (‘recombination, genetic’[MeSH Terms] OR (‘recombination’[All Fields] AND ‘genetic’[All Fields]) OR ‘genetic recombination’[All Fields] OR ‘recombination’[All Fields]) AND recombinant[All Fields]. In total, 96 full-text English-language research articles, either direct PubMed search results hits or identified via perusal of selected articles’ bibliographies, were included in this compilation. Corresponding sequence searches in the NCBI nucleotide database yielded a total of 83 NoV recombinant sequences. Table 1 thus enlarges the scope of intergenotypic recombinant types to 80 in total and notably includes three intergenogroup recombinants.

While Bull et al. rigorously applied three methods, namely phylogenetic analysis, SimPlot analysis and the $\chi^2$ method, to identify recombinants and excluded (certain intergenotypic and all intragenotypic) reported recombinant strains that failed to meet all three criteria, such a detailed recombination analysis would exceed the scope of this review and, accordingly, reported recombinants were preliminarily assumed to be genuine on the strength of their original analysis. Confirmation of the ‘recombinant status’ was sought via the automated Norovirus Genotyping Tool (Version 2.0), available online from the NoroNet website of the Dutch National Institute for Public Health and the Environment (www.rivm.nl/mpf/norovirus/typingtool). Briefly, the tool employs a typing algorithm on ORF1 and ORF2 sequences of genogroup I and II noroviruses, starting with BLAST analysis of the query sequence against a reference set of Caliciviridae sequences. This is followed by phylogenetic analysis of the query sequence and a sub-set of the reference sequences, to assign NoV genotype and/or variant, with profile alignment, construction of phylogenetic trees and bootstrap validation [35]. Interestingly, certain NoroNet analyses yielded divergent results from those published by the original authors, either refuting the submitted sequences’ ‘recombinant’ status entirely or divergently identifying either RdRp- or capsid-sequence affiliation. While this effect probably reflects the regular monitoring and updates of the typing tool’s reference set by the ‘Norovirus working group’ members [this is supported by the fact that most ‘mismatches’ appear in older publications (before 2012)], it also calls into question whether a shared sequencing protocol and stricter baseline limit of nucleotides should be established for analysing and reporting of NoV recombinants [30].

**Intragenotype/intersubtype recombinants – importance in the GII.4 lineage**

Considering that recombination at the ORF1/2 boundary region, as mediated by the proposed homologous copy-choice mechanism, has been associated with high similarities of implicated sequences [33], intragenotype/intersubtype recombination should actually occur more frequently than intergenogroup and intergenotype recombination [41]. Reports of intragenotype recombinants are, however, often difficult to confirm, necessitating extremely sensitive methods to avoid confusion of true intragenotype recombinants with strains that have simply undergone genetic drift. Logically, this liability is much increased in intragenotype recombinants as compared to those derived from more distantly related parental strains [22]. Accordingly, in 2007, Bull et al. were unable to confirm nine reported intragenotype recombinants [34], amongst these notably Hu/NLV/Saitama U3/02/JP and Hu/NLV/GII/MID145-12/87/US (GenBank accession numbers AB039776 and AY032605, respectively) [51]. Surprisingly, the NoroNet Sequencing Tool today identifies the former as a GII.P7/GII.6 recombinant, while the latter groups as a non-recombinant GII.4 Camberwell_1994 in both polymerase and capsid regions.

Nevertheless, intragenotypic recombination has been increasingly cited as an important means for GII.4 (and other) variant emergence and successful navigation of the fitness landscape [52]. The phenomenon has been discussed exhaustively in recent publications [22, 37, 53, 54], but this review would nevertheless not be complete without a short overview of the issue. Postulated mediators for intragenotype GII.4 recombination include selective advantages and improved adaptation to host receptors via physicochemical P2 changes in the virion of new GII.4 subtypes and the evasion of herd immunity against predominant genotypes [29, 41, 42, 55]. Motomura et al. [41] reported evidence of intersubtype genome mosaicism of GII.4 subtypes 2007a, 2007b, 2008a and 2008b, showing them to be ORF1/2 recombinants with distinct evolutionary lineages for capsid- and non-structural proteins of previously co-circulating GII.4 subtypes [40, 41]. NoroNet Sequencing Tool analysis revealed the sequences to be recombinants of GII.Pe/GII.4 Osaka_2004 (GenBank accession number AB541190.1) for subtype 2007a and GII.P4 Apeeldoorn_2007/GII.4 Den_Haag_2006b for the remaining three strains (2007b: AB541193.1 and AB541192.1; 2008a: AB541196.1 and AB541195.1; 2008b: AB541200.1).

Complex patterns of intragenotypic recombination within the GII.4 lineage were revealed with the identification of double and triple recombinant forms involving the P2 antigenic domain of GII.4/2008 variants [42]. The pattern was backed up by detection of interpandemic recombinants between GII.4 New_Orleans_2009 and GII.4 Sydney_2012, in itself a recombinant variant (representative GenBank accession number of a GII.P4 New_Orleans_2009/GII.4
Table 1. Norovirus intergenotype and intergenogroup recombinant types

Recombinant types, as reported by Bull et al. [34] and newly assembled following a systematic PubMed and GenBank search, including literature and database hits published from July 2007 to February 2018. Wherever available, sequences were submitted to the RIVM (NoroNet) Sequence Typing Tool [35] for verification. All recombination breakpoints of recombinants listed in this table are typically located in the vicinity of the ORF1/2 overlap/boundary region, if not otherwise indicated.

<table>
<thead>
<tr>
<th>NoV genogroup</th>
<th>Prototype strain</th>
<th>RdRp genotype</th>
<th>Capsid genotype</th>
<th>GenBank accession no.</th>
<th>RdRp genotype – RIVM (NoroNet) Typing Tool</th>
<th>Capsid genotype – RIVM (NoroNet) Typing Tool</th>
<th>First published</th>
<th>Related strains reported in the literature</th>
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<tr>
<td>1 Hu/GI.4/Beijing/55042/2007/CHN</td>
<td>GLa</td>
<td>GL3</td>
<td>Q856473.1</td>
<td>GL.Pa</td>
<td>GL3</td>
<td>[95]</td>
<td>[59]</td>
<td></td>
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<tr>
<td>2 Hu/GI.4/Beijing/53997/2007/CHN</td>
<td>GLb</td>
<td>GL6</td>
<td>Q856463.1</td>
<td>GL.Pb</td>
<td>GL6</td>
<td>[95]</td>
<td>[59]</td>
<td></td>
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<tr>
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<td>GL2</td>
<td>GL6</td>
<td>AB081723</td>
<td>GL.Pb</td>
<td>GL6</td>
<td>[96]</td>
<td>[97–99]</td>
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<td>GL2</td>
<td>GL8</td>
<td>AB354289.1</td>
<td>GL.Pb</td>
<td>GL6</td>
<td>[96]</td>
<td>[97, 100]</td>
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<td>8 SaitamaT25</td>
<td>GL1.1</td>
<td>GL1.4</td>
<td>AB112100.1</td>
<td>GL.Pd</td>
<td>GL3</td>
<td>[101]</td>
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<td>9 Sydney221298/AU</td>
<td>GLa</td>
<td>GL3</td>
<td>AF190817.1</td>
<td>GL.Pa</td>
<td>GL3</td>
<td>[102]</td>
<td>[33, 103–106]</td>
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<td>GII.P21</td>
<td>GII.1</td>
<td>[33]</td>
<td>[61, 62, 95, 107–111]</td>
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<td>[58, 110]</td>
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<td>AH011002 (formerly AF409067)</td>
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<td>[112]</td>
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<td>unpublished</td>
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<td>N/A</td>
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<td>[108, 110, 124]</td>
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<td>15 Hu/V1628/06/IND†</td>
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<td>GII.7</td>
<td>AB453773.1</td>
<td>GII.P21</td>
<td>GII.7</td>
<td>[125]</td>
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<td>GII.13</td>
<td>FJ922707.1 (RdRp) and FJ986385.1 (capsid)</td>
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<td>GII.13</td>
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<td>[62, 98, 121, 123, 126]</td>
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<td>GII.18</td>
<td>EU921356.1; EU019230</td>
<td>GII.P21 (EU921356.1)/GII.P21 (EU019230)</td>
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<td>[128, 129]</td>
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<td>GII.2</td>
<td>AY134748.1</td>
<td>GII.Pc</td>
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<td>GII.P22</td>
<td>GII.3</td>
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<td>[124]</td>
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<td>GII.5</td>
<td>AB112306.1</td>
<td>GII.P22</td>
<td>GII.5</td>
<td>[101]</td>
<td>[59, 130, 131]</td>
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<td>GII.b</td>
<td>EU921388.2</td>
<td>GII.Pc</td>
<td>GII.4 Osaka, 2007</td>
<td>[128]</td>
<td>[14]‡</td>
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<td>GII.2</td>
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<td>GII.Pe</td>
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<td>JX459908.1</td>
<td>GII.Pe</td>
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<td>GII.12</td>
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<td>[134]</td>
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<td>JF697289.1</td>
<td>GILPg</td>
<td>GII.1</td>
<td>[64]</td>
<td>[28, 29, 43, 56, 58, 59, 75, 131, 133, 135–139]</td>
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<td>GILPg</td>
<td>GII.3</td>
<td>KY442319.1</td>
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<td>GII.12</td>
<td>HQ688986.1</td>
<td>GILPg</td>
<td>GII.12</td>
<td>[139]</td>
<td>[28, 43, 55, 57–59, 95, 123, 130, 135, 138, 140, 141]</td>
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<td>GILg</td>
<td>GII.13</td>
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<td>[65]</td>
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<td>[170, 171]</td>
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Intergenogroup

| I/II | Hu/Kolkata/L8775/2006/IND | GL3 | GII.4 | AB290150.1 | GLP8 | Could not assign | [172] | – |
| I/I | GII/GL isolate AG4 | GII.9 | GII.7 | K662401 (RdRp); K636986 (capsid) | GII.P7 | GII.6 | [154] | – |
| IV/VI | GVI/I/Ca/JPN/2012/M49-1 | GILIV.2 | GILIV.1 | LC011951.1 | GILIV | GII | [94] | – |

N/A: sequence not reported.

Unshaded: novel recombinant types published between July 2007 and February 2018 and verified via the NoroNet Sequence Typing Tool.

Shaded light grey: recombinant types, as reported by Bull et al. and verified via the NoroNet Sequence Typing Tool.

Shaded dark grey: reported (either by Bull et al. or original publication) as recombinant NoV strain but not recombinant according to the NoroNet Sequence Typing Tool.

Shaded blue: sequence confirmed as recombinant but exact ID divergent from initial report; this colour coding does not imply that successively reported sequences do not correspond to their respective recombinant type.

Shaded orange: sequence reported as one of the recombinant types already established by [34], but identified as novel recombinant type via NoroNet Sequence Typing Tool.

Shaded green: sequence (part) not sorted into a defined cluster/recombinant type upon initial reporting but retrospectively identified as constituting a recombinant type.
The strain V1628 (AB453773.1) showed 96% identity in its RdRp region with Pont de Roide 673/FRN (GenBank accession number AY682549), in itself identified as a GII.P21/GII.2 recombinant, *GII.b* (now known as GII.21) was initially described as an obligatory recombinant owing to its typical pairing of a phylogenetically unique ORF1 region with one of a number of genotypes (GII.1, GII.2, GII.3, and GII.4) among other things in the absence of a ORF2 gap phylogenetic cluster [16–18]. When the Italian strain Hu/Pune/PC51/2007/India (GenBank accession number EU921388.2) the recombinant sequence was assigned as GII.e/GII.b [14], whereas the group’s own Sequence Typing Tool (introduced in the cited publication) called a GII.Pe/GII.4 Osaka_2007 recombinant upon analysis in January 2018 (10/01/2018). This result probably reflects the regular monitoring and updates of the typing tool’s reference set by the norovirus working group members.

‡Interestingly, when Kroneman et al. [14] typed the strain Hu/Pune/PC51/2007/India (GenBank accession number EU921388.2) the recombinant sequence was assigned as GII.e/GII.b [14], whereas a putative GII.4 intragenotype recombinant, sporting an atypical recombination event between ORFs 2 (new GII.4 variant) and 3 (Den Haag subcluster) (GenBank accession number EU921388) was identified in 2010 via full-genome sequencing using overlapping primer sets [39]. The NoroNet Sequencing Tool, however, which does not use ORF3 sequences, identifies the sequence as GII.Pe/GII.4 Osaka_2007 recombinant (supported with phylogenetic analysis and bootstrap 100 (≥70.0)). Intragenotype recombinants, GII.4 US95_96/GII.4 Kaiso_2003 (GenBank accession numbers KU756290–KU756293) and GII.4 Den_Haag-2006b/GII.4 Yerseke_2006a/GII.4 Apeldoorn_2007 were recently reported in South Africa [54]. The recombinant GII.4 strain Cape Town 6745 (GenBank accession number KJ710245.2) [58], with a *New Orleans* 2009 polymerase, still remains unassigned in its capsid sequence.

A putative GII.4 intragenotype recombinant, sporting an atypical recombination event between ORFs 2 (new GII.4 variant) and 3 (Den Haag subcluster) (GenBank accession number EU921388) was identified in 2010 via full-genome sequencing using overlapping primer sets [39]. The NoroNet Sequencing Tool, however, which does not use ORF3 sequences, identifies the sequence as GII.Pe/GII.4 Osaka_2007 recombinant (supported with phylogenetic analysis and bootstrap 100 (≥70.0)). Intragenotype recombinants, GII.4 US95_96/GII.4 Kaiso_2003 (GenBank accession numbers KU756290–KU756293) and GII.4 Den_Haag-2006b/GII.4 Yerseke_2006a/GII.4 Apeldoorn_2007 were recently reported in South Africa [54]. The recombinant GII.4 strain Cape Town 6745 (GenBank accession number KJ710245.2) [58], with a *New Orleans* 2009 polymerase, still remains unassigned in its capsid sequence.

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Both typical and atypical intragenotype recombinant thus contribute to the growing complexity of the GII.4 lineage, furthering GII.4 variant emergence and spread.

### Obligatory norovirus recombinants – the ‘odd ones out’ in an unsolved and never-ending Rubik’s cube?

While a NoV recombinant is typically comprised of two (or more) sections of different ORF1 and ORF2 genotypes which are paired in the respective parental strains at either side of the typical recombination breakpoint, several exceptions exist. A number of polymerase types, such as GII.a, c, e and n, have been assigned preliminary letter-based names rather than genotype numbers to signify that only their ORF1 sequences have been detected with no known ‘own’ capsid sequences [14]. Such ‘orphan’ ORF1 genotypes can be promiscuously associated with capsids of different genotypes. As such, GII.b (now known as GII.P21) was initially described as an obligatory recombinant owing to its typical pairing of a phylogenetically unique ORF1 region with one of a number of genotypes (GII.1, GII.2, GII.3 and GII.4).
amongst others) in the absence of an ORF2 GII.b phylogenetic cluster [61, 62]. Its status was only changed when the Indian strain Hu/NoV/Ahm PC03/2006/India (GenBank accession number EU019230) was identified as present in the partial ORF (pORF) GII.21 cluster together with representatives of the pORF1 type II.b. The orphan GII.b cluster was consequently renamed GII.21 [14]. First emerging in 2008 in Victoria, Australia, the obligatory recombinant GII.e (identified by ORF1 nucleotide sequencing) became the prominent ORF1 genotype in 2012, superseding GII.4 [27]. No unique GII.e ORF2 genotype has been identified and GII.e continues to cluster with a multitude of ORF2 sequences (amongst these GII.3, GII.12 and various GII.4 variants) [27, 56, 63, 64]. The obligatory NoV recombinant GII.Pg, already present in 1989 [65], re-emerged clinically around the same time as GII.Pe, and has so far been confidently associated with the four different ORF2 genotypes, GII.1, GII.3, GII.12 and GII.13 [28, 64, 66].

Similarly to the obligatory recombinant ORF1 genotypes, other polymerase genotypes, notably GII.4, GII.7, GII.12, GII.16 and GII.21 have been reported to be associated to more than one capsid genotype, supporting the polymerase as the driving factor in recombination [34, 39, 57, 64]. While the reasons for this are unclear, poor processivity of certain polymerases, rendering an ORF1/2 template switch more likely, has been advanced as a possible factor [34]. Polymerase fidelity has been identified as the determining factor in driving recombination in other RNA viruses [67] and should also be considered in this context. Inversely, but less frequently, certain capsid types (e.g. GII.3) have been associated with multiple polymerases. A quantitative representation, showing intragenogroup association of NoV polymerases to genetically diverse capsid types, is provided in Fig. 2.

It has been noted that ‘the fact that a virus has an identical VP1 and pORF2 type does not necessarily mean that it is not a recombinant’ [14]. It seems, that in the ever-changing, ever-shifting association of NoV capsid and polymerase types, it is near-impossible to clearly say ’who originally belonged to whom’. This begs the question whether NoV classification, necessarily based on a transversal, arbitrary cut-off, indeed correctly associates capsids and polymerases, or whether ’parental’ strains are in themselves recombinants. We can liken this to an unsolvable Rubik’s cube, in which matching colour codes do not necessarily signify togetherness and in which each rotatable single square may be pivoted to partner with any of the other squares.

**NOROVIRUS RECOMBINATION IN THE LAB – A RECAP OF IN VITRO AND IN VIVO STUDIES**

Few experimental data are available concerning NoV recombination under laboratory conditions. The first *in vitro* experimental evidence of NoV recombination, was obtained for MuNoV (GV), using a PCR-based discriminating tool, to demonstrate a homologous recombination event at the ORF1/ORF2 overlap of the MuNoV genome [68]. In this study, cell cultures were co-infected with two parental homologous MuNoV strains CW1 and WU20, sharing an 87 % nucleotide sequence similarity in their complete genomes, and a single viable recombinant virus was detected and isolated from an infectious centre assay. This recombinant has since been shown to display similar biological properties to its parental strains *in vivo*, albeit with a slight reduction in replicative fitness [69]. The first artefact-free estimate of *in vitro* recombination rates ($P_{rec}$) between these same two strains co-infecting a murine macrophage culture (at high m.o.i.:2) was obtained via use of drop-based microfluidics [70]. The $P_{rec}$ of co-infecting progeny viruses was measured as $3.3 \times 10^{-4} \pm 2 \times 10^{-5}$; however, it remains undetermined whether the rare RNA recombinants identified were indeed viable infectious MuNoV recombinants. Recently, we examined whether different parameters of co- and superinfection, prerequisites for recombination events, influence the frequency of recombination *in vitro*. No viable recombinants could be detected after synchronous and asynchronous infections of cultured murine macrophage cells with the two homologous MuNoV strains WU20 and CW1 using different multiplicities and different times of infection (as yet unpublished preliminary results). The phenomenon of NoV recombination is not easily reproducible in laboratory conditions, and has been shown to be apparently rare both *in vitro* [68, 70] and in a recent *in vivo* study [44], where MuNoV recombinants were isolated from CW1- and WU20 co-infected mice. Interestingly, in addition to the typical ORF1/ORF2 breakpoint, Zhang et al. [44] also detected recombination events with low frequencies in the VPg, protease and 3’end of the RdRp coding region as well as the VP1 S domain of MuNoVs. These newly detected recombinants were, to our knowledge, again not tested for infectivity and it is uncertain, whether the identified additional breakpoints would generate replication-effective recombinants, as virus-amplification steps did not succeed their identification. This somewhat limits the extent to which reliability can be placed upon the occurrence of such a recombination event in a replication-effective MuNoV.

Compared to the sheer quantity of reports of HuNoV recombinants detected in the field, the paucity of information regarding MuNoV recombination as studied in the lab either *in vitro* or even *in vivo*, is evident. Equally, the difficulties accompanying the generation of a recombinant MuNoV *in vitro* [68], stand in stark contrast to the comparable ease of generating recombinants of other more distantly related [71] or even very similar [72] viruses under comparable circumstances. The only epidemiological data available for MuNoVs are the early screening results of 76 faecal samples from 28 different SPF mouse lines which suggested intergenotypic recombination events for MuNoVs [73]. Comparisons of the vastly different-sized datasets for HuNoVs and MuNoVs and their corresponding denominators allow only very limited inferences and, due to a lack of data, no conclusions can be drawn at this time regarding a comparison of recombination frequencies in the field and under laboratory conditions.
Fig. 2. Intragenogroup association of norovirus polymerases to genetically diverse capsid types. The figure shows the association of norovirus polymerase types (far left column) with different capsid types (top row) and vice versa for genogroups GI (above) and GII (below). The total number of genetically diverse capsid types (∑capsid) to which a polymerase type within the same genogroup is associated, is given in the far right column. The total number of genetically diverse polymerase (RdRp) types (∑RdRp) to which a capsid type within the same genogroup is associated, is given in the bottom row. Known ‘obligatory recombinants’ GII.Pe and GII.Pg are shaded in red tones. Green squares mark spatial positions of strains with ‘matching’ polymerase and capsid types (e.g. GII.P4/GII.4) as reported by Vinjé [13].
DRIVERS AND CONSTRAINTS OF NOROVIRUS RECOMBINATION

Loosely based on a pre-existing model for the production of a viable recombinant RNA virus [74], the necessary steps, including their respective drivers and constraints (predictive risk factors), for recombination of NoVs are considered (Fig. 3). Five steps must be successfully passed to generate a viable, replicating recombinant NoV following the classical copy-choice model of replicative NoV recombination. We suggest four steps to obtain the same result via non-replicative recombination, the main difference being the process (and enzymes) needed for joining partial parental sequences, once in close proximity within a cell.

The first (simplified) step necessarily preceding any recombination event is the simultaneous infection (co-infection) of a host with at least two parental strains (Fig. 3a). The ability of different NoV strains to overlap sufficiently in space and time to effect co-infection of a host is undoubted. Not only are several strains frequently detected to be co-circulating within the context of a single outbreak but also within a single patient. Only recently, e.g. circulation of GII.4, Pg/GII.1 and GII.Pe/GII.4 Sydney 2012 recombinant variants was detected in an asymptomatic population in Indonesia. Of seven positive individuals, two were repeatedly infected with the same strain and heterogenous strains [75]. Both the noroviruses’ notorious low-level antigenicity between strains [76] as well as the well-documented delayed immune clearance, genetic diversity and continuing quasispecies evolution in immunocompromised patients [77-79] facilitate host co-infection. Further opportunities for human co-infection present themselves via a typical mode of NoV infection, food- or waterborne outbreaks, and in particular the consumption of bivalve molluscs, which have been known to accumulate several different strains in their intestines [80, 81]. An overview of NoV transmission routes, highlighting the three above-mentioned ’hot-spots’ for accumulation of multiple NoV strains and increased risk of viral recombination is presented in Fig. 4.

Once a host has been successfully co-infected, the second step is co-infection of a single cell. While factors such as a strong host immune response or virus-mediated superinfection exclusion might prevent this, novel models of NoV pathology and cell-tropism present intriguing mechanisms for bypassing these barriers. Karst and Wobus compellingly suggest that NoVs, bound to motile bacteria and/or host carbohydrates in the gut lumen, could be taken up via Peyer’s patch-associated M-cells to then be delivered to permissive immune cells in the underlying lamina propria [82]. Not only may the simultaneous uptake of multiple viruses into a single cell be possible via this route, but also a putative subsequent persistent infection of target cell immune cells could be seen to heighten chances of co-infection at a later date.

The third step to obtaining a recombinant virus can either be a step of non-replicative recombination in which viral genome fragments are ligated by host factors (Fig. 3b) [46] or, as described for NoVs above, a combination of replication and template switch (step four), between two co-infecting viruses within a cell (Fig. 3c).

By whichever way a recombinant NoV genome is created, it does not necessarily follow that this will yield a replicating recombinant NoV. Any given recombination event, switching out large genome parts in a nascent virus, presents a significant modification. It follows, that initial imprecise recombination events (e.g. introduction of mutations or faulty epistatic interrelationships between the two parts of a novel recombinant) present an evolutionary bottle-neck which can either result in non-functional genome recombinants or in reduced-fitness recombinants. The droplet or fomite transmission of NoVs, their low infectious dose [83] and typically observed high viral loads [80] entail that even poorly performing recombinants may get an opportunity to survive, to resolve and then to proliferate in the viral population at a between-host level. If recombinants do not possess selective advantages over their parental strains, it is unlikely that they will be maintained in a viral population within the original host before they can undergo a stage of resolution optimizing their replicative fitness [45, 84]. The typical ORF1/2 recombination breakpoint of NoVs indicates how recombinants may ‘survive’ this fifth step, selection. Such a recombination event may confer advantages under host- or population-level immune pressure that can outweigh initial detriments to replicative fitness. Putative replicative disadvantages, it seems, are more than compensated for by other advantages at the level of competitive or transmissive fitness, when a ‘coat switching’ event occurs in which a novel recombinant couples non-structural proteins from one and structural proteins carrying antigenic determinants from the other parental virus [33, 39, 85, 86].

TRUE RECOMBINATION OR RAPID GENETIC DRIFT?

Detection of viral recombination events at a population level in the field is traditionally based on bioinformatical analyses, implementing similarity, distance, phylogenetic and compatibility methods and/or substitution distribution [17]. When applied to positive-sense RNA viruses with high mutation rates [87], a pitfall of all recombination detection programmes is the possibility of overestimating the frequency of genetic shift. In other words, phylogenetic-based analyses of recombination can be affected by convergent evolution leading to similar sets of nucleotide and amino acid substitutions in independent lineages [42]. For NoVs, mutation rates have been inferred to correspond to a rate of 2–9×10⁻³ substitutions per nucleotide per year [88]. Hoffmann et al. [89] demonstrated that GII.4 and GII.7 strains underwent positive selection during chronic infection of immunocompromised patients at an even more elevated evolutionary rate as compared to that found at an inter-host population level (owing to constant intra-individual selection pressure) [89]. It seems probable that this
process, whereby NoV strains can acquire enough mutations to constitute novel epidemic subtypes within weeks to months (on a global scale this would normally take years), might contribute to the overestimation of recombination ('false positive' identification of NoV recombinants), when a fast genetic drift is mistaken for recombination. The solving of the Rubik's cube, it seems, can be further muddled by squares changing their colours while the puzzle is being pivoted.

**NOROVIRUS RECOMBINATION, INTERSPECIES TRANSMISSION AND ZOONOSIS RISK**

The as yet unproven existence of a zoonotic potential for NoVs has long been discussed, potential interfaces of shared species exposure being food, water or animal contact. The discussion about interspecies and/or zoonotic transmission is fuelled by the close relationship of certain animal and human NoV strains, detection of HuNoVs in animal faeces, detection of antibodies against HuNoVs in

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**Fig. 3.** Steps, drivers and constraints of NoV recombination as adapted from Worobey and Holmes [74]. (a) gives an overview of the different recombination steps and accompanying host, virus or environmental drivers and constraints (predictive risk factors). (b) and (c) focus on putative drivers and constraints of both non-replicative recombination and template-switch-mediated recombination [replication and template switch via the RdRp (NS7)], respectively. APC: antigen-presenting cells; p.i.: persistently infected.
swine and the demonstration of experimental HuNoV GII infection in gnotobiotic pigs [2, 90–92]. Questions concerning species barrier determinants preventing HuNoV infection of murine cells were recently resolved with the identification of a CD300lf proteinaceous receptor as the primary determinant of MuNoV species tropism, showing other components of cellular machinery required for NoV replication to be conserved between humans and mice [93]. If we assume the key to cross-species transmission to be located only at a structural virus-host receptor level, this presents ORF1/2 NoV recombination, by which a nascent recombinant virus gains a complete novel capsid protein set, in an interesting light, in that a ‘lucky’ intragenogroup recombination event might tender a zoonotic/interspecies recombinant. Indeed, putative GIV.2_GVI.1 interspecies recombinant FNoVM49 isolated from a cat captured near a Japanese oyster farm in 2015 [94], may have originated via a similar mechanism.

CONCLUSION AND UNANSWERED QUESTIONS

Recombination, shifting the ‘Rubik’s cube’s building blocks’ of NoV classification, remains a significant factor influencing NoV molecular evolution and diversity. The enormous scope of intragenotype, intergenotype and even intergenogroup NoV recombinants and their recurrent implication in reported outbreaks highlight the continued importance of standardized monitoring (via shared sequencing protocols or implementation of whole-genome next-generation sequencing) and reporting of novel NoV recombinant types. With respect to their potential to emerge and re-emerge as dominant NoV strains, early detection of NoV recombinants and an understanding of the possible impact of recombination on (future) vaccine usage must be furthered. Special attention should be paid to recombination between genetically distant NoVs, which may generate novel NoV variants with altered pathogenesis and modified host tropism.

Despite the abundance of epidemiological data recording different, mainly HuNoV, recombinant types, evidence for MuNoV recombinants generated in vitro is scarce and the mechanism(s) involved are poorly characterized. It remains to be seen whether there is a true disconnect between NoV recombination frequency in the field and its apparent rarity under laboratory conditions. Since the MuNoV model allows only limited inferences regarding NoV recombination, in vitro HuNoV recombination studies in robust cell culture systems, the development of novel tools (NGS analysis of RNA within the cell and improved reverse genetic systems) to allow generation and detection of recombinants, as well as co-infection studies with other animal models, will

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**Fig. 4.** Norovirus transmission and dispersion routes and hotspots for co-contamination and co-infection with multiple strains. ‘Hotspots’ for the accumulation of multiple norovirus strains are ringed in red, highlighting stagnant waters, tap waters, sewage and bivalve molluscs that can be contaminated with multiple norovirus strains, as well as immunocompromised persons, which typically harbour a diverse norovirus quasispecies. Immunocompromised animals should be viewed in the same light as immunocompromised people but have not been included to maintain simplicity of the figure.
help resolve as yet unanswered questions in this area. In this context, drivers and constraints of NoV recombination must be investigated.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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