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Norovirus vaccines and potential antinorovirus drugs: recent advances and future perspectives

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Abstract

Human noroviruses (HuNoVs) are a leading cause of acute, nonbacterial gastroenteritis worldwide. The lack of a cell culture system and smaller animal model has delayed the development and commercial availability of vaccines and antiviral drugs. Current vaccines rely on recombinant capsid proteins, such as P particles and virus-like particles (VLPs), which have been promising in clinical trials. Anti-HuNoV drug development is another area of extensive research, including currently available antiviral drugs for other viral pathogens. This review will provide an overview of recent advances in vaccine and antiviral development. The implication of recent advances in HuNoV cell culture for improving vaccine and antiviral development is also discussed.

Keywords

antivirals; human norovirus; vaccines

Noroviruses (NoVs) are nonenveloped, positive-sense single-stranded RNA viruses belonging to the *Caliciviridae* family. NoVs are divided into six genogroups (GI–GVI) [1,2]. The GI and GII genogroups are the most important for human infection, but are believed to lack common neutralization epitopes due to major antigenic differences between them. Human NoVs (HuNoVs) are a leading cause of nonbacterial, acute gastroenteritis worldwide and GII.4 HuNoVs account for approximately 60–90% of all HuNoV gastroenteritis annually [3]. HuNoVs are responsible for approximately 21–23 million gastroenteritis cases and 800 deaths in the USA [4] and over 218,000 deaths in developing nations annually, mostly in children less of 5 years of age [5].

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HuNoV infection can occur year round, but has a distinct winter seasonality in temperate climates, earning the nickname ‘winter vomiting disease’ [4]. HuNoVs have an incubation period of approximately 24 h [6] and a disease length of approximately 24–72 h [7]. The most common symptoms are nausea, vomiting and diarrhea, but can also include abdominal cramps, fever, headache and dehydration [8,9]. NoVs are primarily transmitted via the fecal-oral route [8], but can also be transmitted via aerosolized vomitus droplets [10], contaminated food or water [11] and fomites [12]. HuNoVs are easily transmitted in semiclosed units, such as cruise and naval ships and senior care facilities. The young and the elderly are more commonly infected and more prone to severe disease outcomes, respectively [11].

Despite years of attempts, no independently validated cell culture systems or small animal models have been established for HuNoVs. These limitations have hindered the development of live attenuated or inactivated HuNoV vaccines, antivirals and diagnostic assays. This review will cover recent vaccine and antiviral development against HuNoV-induced gastroenteritis. HuNoV vaccine candidates have depended upon recombinant capsid proteins, primarily virus-like particles (VLPs) and P particles. HuNoV antiviral development is another area of intense research, including previously examined broad antiviral compounds.

Norovirus epidemiology & economic burden: the need for vaccines & antivirals

The worldwide epidemiology and economic burden of HuNoV gastroenteritis remains unknown, but regional studies and a recent large-scale systemic review and meta-analysis have provided estimates [13]. An excellent review was recently published detailing HuNoV epidemiology [14]. HuNoVs have replaced rotavirus as the most common cause of acute gastroenteritis in countries where rotavirus vaccines are implemented [15,16]. For example, among Nicaraguan children who are vaccinated against rotavirus, HuNoV was the most commonly detected pathogen in all diarrhea samples and the most prevalent pathogen in children <2 years of age [15].

The worldwide economic burden of HuNoV gastroenteritis is also high. Total acute gastroenteritis accounted for approximately $3.88 billion from 2006 to 2011 worldwide [16]. In the USA, HuNoV gastroenteritis resulted in $180 to $355 million in total healthcare costs from 2006 to 2011 [16] and $2 billion in total economic burden annually [17]. A systematic review by the CDC analyzing 175 publications covering 48 countries and 25 years concluded that HuNoV was responsible for 18% cases of all gastroenteritis worldwide and 14–19% in developing countries and 20% in developed nations [13]. Though infections in the young and old are believed to be under-reported [18], these estimates are higher than previously thought, further highlighting the need for HuNoV vaccines and antivirals.

Computer models have shown that a vaccine with 50% efficacy could prevent up to 2.2 million cases annually and reduce HuNoV economic burden by $2.1 billion over 4 years in the USA [19]. The same models estimated that the primary beneficiaries of HuNoV vaccines would be children under the age of 5 and the elderly over 65 years of age [19], which

correspond to the finding that these two age groups are prone to more cases and more severe outcomes, respectively [11]. Based on these estimates, the beneficial economic impact of a HuNoV vaccine is apparent. However, the development of a global HuNoV reporting system will provide clearer information regarding global burden and economic benefits of vaccination.

Function of HuNoV proteins

The HuNoV genome is 7.5–8.0 kb with a 5′ VPg protein cap, 3′ polyadenylated tail and three open reading frames (ORFs) [20], while the murine norovirus (MNV) genome contains four [21]. ORF1 encodes a nonstructural polyprotein, while ORF2 and ORF3 encode the major structural protein, VP1 and minor structural protein, VP2, respectively [20,22]. The MNV ORF4 encodes an innate immune regulatory factor, VF1 [21]. The polyprotein is cleaved at five cleavage sites, yielding six proteins: p48 (NS1/2), helicase (NS3-NTPase), p22 (NS4), VPg (NS5), protease (NS6pro) and RNA-dependent RNA polymerase (NS7pol) [23,24].

The role of HuNoV proteins and their roles in viral pathogenesis and replication was recently reviewed [25] and a mechanism of infection of the intestine has been proposed [26]. The HuNoV p48 is associated with disassembly of the Golgi and impaired protein trafficking [27]. The HuNoV NTPase was previously identified as p41 and is similar to the picornaviral 2C protein. The HuNoV NTPase/helicase binds ATP and GTP, but does not exhibit typical helicase activity [28]. The HuNoV p22 is also associated with fragmented Golgi and inhibited cellular protein secretion, presumably through reduced vesicle trafficking from the ER to the Golgi [29]. Caliciviruses do not contain a 5′ cap or internal ribosomal entry site on their genome, but instead have a protein covalently linked to the genome, VPg [30]. Studies on HuNoV VPg have remained limited as the lack of a cell culture system restricts the amount of available VPg protein. One study indicated that the C-terminal domain of VPg primarily binds host initiation factors and facilitates recruitment of ribosomes for translation, but do not inhibit host protein translation [31]. Furthermore, a more recent study indicates that MNV infection can induce phosphorylation of host initiation factors, such as eIF4E, and regulate translation of host mRNAs [32].

The HuNoV protease cleaves at five highly conserved cleavage junctions: Q^{330}/G^{331}, Q^{696}/G^{697}, E^{875}/G^{876}, E^{1008}/A^{1009} and E^{1189}/G^{1190} [24]. An *in vitro* study indicated that the Q–G sites are cotranslationally cleaved first, releasing p48, the NTPase/helicase and a p22/VPg/protease/polymerase complex [24]. This complex is further processed to p22/VPg and protease/polymerase, but likely requires host factors for catalysis [24]. The HuNoV genome encodes an RdRp, which is difficult to study also due to the lack of a cell culture system. While all NoV proteins can be potential antiviral targets, the most commonly investigated have been the viral RdRp and protease.

VP1 and VP2 are the major and minor structural capsid proteins, respectively [33,34]. The VP1 capsid protein consists of the shell (S) domain and the protruding (P) domain; the two are connected by a hinge [35]. The P domain is further divided into two subdomains, P1 and P2 [33]. VP2 increases capsid stability and interacts with the S domain of VP1 [36,37].

Genomic factors in HuNoV immunity

NoV is a highly infectious virus with as few as 18 viral particles able to cause infection [38]. We are still learning of the host side risk factors associated with HuNoV infection, disease and transmission. A sample of host factors that have been clearly understood in HuNoV susceptibility are histo-blood group antigen (HBGA) type, secretor status, age and immune status (Table 1). HBGAs are a receptor for HuNoVs [39]; other receptors or coreceptors may also exist. Tan and Jiang comprehensively reviewed the role of HBGAs in HuNoV infection in a recent article [39]. HBGAs are complex carbohydrate moieties expressed on red blood cells, gastrointestinal, genitourinary and respiratory mucosal epithelial cells, and in biological fluids as free oligosaccharides [40]. HGBA biosynthesis is catalyzed by glycosyltransferases in three gene families: the ABO, secretor and Lewis that encode A and B enzymes, FUT2 and FUT3, respectively [39]. FUT2 is the enzyme necessary for the synthesis of H or secretor antigens [41]. Whether FUT2 gene is functional is the key for the most well-recognized risk factors for HuNoV infection, i.e., HBGA type O [42] and secretor-positive status [43]. Individuals with nonfunctional FUT2 gene are called nonsecretors and they have reduced susceptibility to HuNoVs.

In addition to HGBA, other cellular cofactors may also play a role in viral binding, entry and uncoating. For example, MNVs bind to sialic acids and glycolipids [46,47] and recent studies suggest that enteric bacteria bind HuNoV [48] and can increase HuNoV infectivity *in vitro* [49]. HuNoV–HBGA binding is mediated by a binding pocket with a conserved motif surrounded by strain-specific amino acids [50]. The binding patterns of HuNoVs to HBGAs are strain-specific [43,44]. For example, individuals with HBGA type O are more susceptible to GI.1 Norwalk virus infection [41]. A study of HuNoV-infected Vietnamese children revealed all GII.4 cases involved secretors (H1+ Lewis b and/or Lewis y HBGAs) or partial secretors (Lewis a and b or Lewis x and y HBGAs), but not non-secretors [43]. However, there were five cases of nonsecretors infected with GII.3 HuNoV [43]. Another recent study indicates GII.4 HuNoV infection is correlated with positive secretor status, while non-GII.4 strains were associated with nonsecretors among Ecuadorian children [51]. Further global studies are needed to determine host factors that determine HuNoV susceptibility since conclusions from a regional study cannot always be extrapolated to all populations. Particular attention should be paid to differences in genetic factors as a recent study indicates ancestry impacts HuNoV susceptibility and secretor genotype varies by genetic ancestry and ethnicity [52].

On the virus side, there are 40 genotypes of HuNoVs in genogroups GI and GII (11 GI and 29 GII) and many variants in each genotype, particularly in GII.4. Novel HuNoV variants emerge from host immune evasion [53] and potentially from persistence in immunocompromised patients [54]. The tremendous viral diversity makes the development of broadly protective HuNoV vaccines very challenging. However, mathematical modeling studies of community HuNoV transmission suggest that protection from a natural infection lasts 4.1–8.7 years [55], though previous challenge studies in humans showed susceptibility as early as 27 months after an infection [56].

B cells in HuNoV immunity

Antibodies and B cells have been shown to be important for HuNoV immunity, but B cells may also be necessary for HuNoV replication. B-cell immune responses in HuNoV infections were recently reviewed [57,58]. First, serum HBGA blocking antibodies have been identified as a correlate of protective immunity against NoV-induced disease [59,60]. A more recent study has indicated that virus-specific salivary IgA antibodies and circulating IgG secreting memory B cells are also correlates of protection, though only memory B cells are capable of persisting for 180 days postinfection [61]. These antibodies primarily recognize the P2 domain of the VP1 capsid protein [62], but these sites are regulated by several factors, including viral particle confirmation, temperature and external amino acid residues [63]. Antibody responses are associated with decreased viral shedding, clearance of MNV infection and prevention of viremia [61,64–65]. Specifically, fecal IgA has been correlated with decreased viral titers [61]. Limited human studies have confirmed that HuNoV infection induces serum IgG antibody responses [66] and IgA-biased antibody-secreting cells and IgG-biased memory B cells [61]. Additionally, serum IgG was detected following infection of gnotobiotic (Gn) calves with GIII.2 bovine NoVs [67]. HuNoVs and MNVs were recently shown to infect B cells *in vitro* [49], but have not been independently validated. Direct infection of B cells by NoV may partially explain the weak antibody responses and limited long-term immunity following natural NoV infection. Studies should continue to elucidate the primary B-cell populations associated with protective immunity as well as those that may be infected by HuNoVs.

T cells in HuNoV immunity

The understanding of HuNoV-induced cellular immunity remains limited, though there have been several studies analyzing NoV-induced T-cell responses in animal models and humans. The current understanding of T-cell immune responses following HuNoV infection was recently reviewed [57,58]. Infection of macrophage cells with MNVs resulted in the expression of pro-Th1 chemokines [68]. C57BL/6 mice infected with MNV CW3 (acute infection) or MNV CR6 (persistent infection) resulted in differential T-cell patterns; CR6-infected mice had fewer functional $CD8^+$ T cells than CW3-infected mice [69]. Similarly, $CD8^+$ T cells decreased viral titers in Rag1(−/−) mice, implicating a role of $CD8^+$ T cells in clearance of viral infection [69]. Importantly, $CD4^+$ T cells, but not IFN-γ, have been shown to be a correlate of protection from MNV infection [70], although IFN-γ has been detected in serum and lymphoid tissues following HuNoV challenge in humans [66]. HuNoV infection increases $CD8^+$IFN-γ^+ T cells and Tregs in duodenum of Gn pigs at 7 days postinoculation [71]. Together, these results indicate that HuNoV induces a predominant, yet weak, Th1 response along with a strong Treg response. Early challenge studies showed that HuNoV infection induces short-term, homologous protection [56,72]. Humans challenged with Norwalk virus were able to be re-infected 42 months later [56]. The strong Treg response observed in Gn pigs may downregulate the effector T-cell responses and prevent the development of memory cells, leading to the short-term immunity. Due to the time limit of keeping Gn pigs in isolators, the duration of long-term protection cannot be evaluated in Gn pigs. Differential modulation of the Th1 and Treg responses may be critical to the efficacy and duration of HuNoV vaccine-induced protection. The key responding T

cell subsets that are responsible for protective immunity remain unknown and are an important target for future studies.

VLPs vaccines

Potential vaccines against HuNoVs have been a main area of research since the discovery of Norwalk virus in 1972. Due to the inability to culture the virus, vaccine development has relied upon recombinant capsid proteins, including VLPs and P particles. A summary of these vaccine candidates is presented in Table 2. VLPs have been extensively studied as HuNoV vaccine candidates [73–75] and several formulations have gone through clinical trials [76–80]. There is an excellent review on the recent advances in VLP vaccine development by Tan and Jiang [81]. VLPs are derived by expression of the VP1 capsid protein in eukaryotic expression vectors and produce a capsid similar to the native virion [82]. High quantities of VLPs can be produced in multiple expression systems, such as baculovirus [82], yeast [83] and plants [84]. As such, VLPs are nonreplicating and retain similar binding properties as wild-type HuNoV [85–88].

Preclinically, VLPs have been studied in mice [94,95] and other animal models including Gn pigs, rabbits and chimpanzees [93,109–110]. In separate studies, GII.4-derived VLPs provided partial protection against diarrhea following cross-variant or homo-variant challenge in Gn pigs [71,109]. Although new variants of GII.4 emerge at approximately 2–4 year intervals [96], HuNoV VLPs can induce genotype-specific cross-variant immunity and partial protection against viruses isolated 8 years apart (from 1998 to 2006) and across the emergence of as many as four variants [71].

The most promising VLP vaccine studies have focused on vaccine formulations that aim to combat HuNoV diversity. For instance, chimeric VLPs expressing the immunodominant epitope A from historical strains of HuNoV provided homotypic and heterotypic antibody responses compared with single-strain VLPs preparations in mice, but weaker blockade responses than single-strain VLPs against their parental strains [95]. Similarly, intramuscular-administered VLPs derived from a consensus GII.4 sequence and Norwalk virus with Alhydrogel adjuvant induced broad antibody responses against the native viruses and other variants in rabbits, though these responses were genotype-specific [93]. Additionally, VLP cocktails have coexpressed GII.4 HuNoV VP1 with rotavirus VP6 antigen [97]. In mice, the combined vaccine induced cross-reactive antibody responses to both pathogens without interfering with the overall immune response [97]. The protective efficacy of these chimeric VLP preparations and HuNoV-rotavirus combination vaccines should be evaluated in large animal models.

VLP regimens consisting of different formulations and routes of administration have undergone or are currently going through human clinical trials and have been evaluated for both immunogenicity and protective efficacy in healthy adult humans by LigoCyte (acquired by Takeda Pharmaceuticals in 2012) [76–78,80]. Intranasal (IN) administration of two doses of Norwalk-derived VLPs provided partial protection against infection (25.6%) and disease (46%) [76] and elicited virus-specific intestinal homing antibodies [77] and memory B cells [78]. More recently, a two-dose 50 μg intramuscular GI.1 plus GII.4 consensus VLP bivalent vaccine reduced occurrence of diarrhea and vomiting (68% reduction for moderate to severe

and 47% for any severity) [79] and increased virus-specific total serum antibodies within 7 days after a single dose in adult humans [80]. However, there was no detectable increase in antibody titers following the second vaccination. The immune response profile suggests that the intramuscular vaccine boosted the anamnestic immune responses in previously HuNoV-infected hosts. Since there is a high prevalence of natural HuNoV infection in all susceptible human populations [98], the intramuscular vaccine approach is likely to be effective as a booster vaccine in adults who have been previously infected and have antibody titers below protection levels. For HuNoV-naive pediatric populations, mucosal vaccines will likely be needed. Overall, VLPs are appealing vaccine candidates and provide a malleable backbone for efficient vaccine design against emerging and recombinant NoV strains.

Vectored VLP vaccines

An alternative strategy to produce VLPs utilizes viral vectors, such as vesicular stomatitis virus (VSV) [99,100], avian paramyxoviruses (Newcastle disease virus, NDV) [102], adenovirus [111] and Venezuelan equine encephalitis virus (VEEV) [103]. Recombinant viral vectors are appealing as they likely require only a single dose and inoculate the host with higher amounts of VLPs than conventional VLP preparations. However, biosafety concerns and preexisting host immunity may limit the development, availability and efficacy of the vector-based vaccine candidates.

VLPs expressed from VSV have been examined in mice [99,100]. Insertion of HuNoV VP1 in VSV attenuated viral growth *in vitro* and *in vivo* [100]. VSV-VP1 (combined IN and oral) inoculated mice experienced severe weight loss, suggesting this vaccine vector system requires further attenuation [100]. Coexpression of HSP70 further attenuated VSV in mice, but did not prevent spread of VSV to the CNS [99]. HSP70 likely induced this attenuation by simultaneously stimulating antiviral interferons and suppressing transcription of downstream VSV genes [99]. Both VSV preparations induced serum, cellular and humoral responses, though VSV-HSP70-VP1 required increased doses for cellular and humoral responses [99,100]. Modified recombinant NDV LaSota (rNDV)-vectored VLP vaccines have also been evaluated in mice recently [102]. The modified NDV-VP1 vaccine induced more robust immune responses, including increased levels of serum IgG, compared with the conventional NDV-VP1 vector and baculovirus-derived VLPs and higher fecal IgA levels compared with baculovirus-derived VLPs. Furthermore, the modified NDV-VP1 vaccine induced splenic IFN-γ, IL-2 and TNF-α secreting cell responses [102].

There are several potential advantages of rNDV-vectored vaccines over VSV-vectored vaccines. First, NDV is a strong stimulator of the mucosal and systemic immune responses. The most common route of natural NDV infection is by the oral route, so it may be possible to inoculate a NDV-vectored HuNoV vaccine orally, which will induce higher level of mucosal immunity in the gastrointestinal tract. In addition, rNDV can produce VLPs in large quantities in embryonated chicken eggs, which would be cost-effective and feasible for large-scale manufacturing of VLP vaccines. Efficient production of VLPs in embryonated eggs can also facilitate the formulation of multivalent VLP-based HuNoV vaccines, which is needed to induce a broad-protective immune response. However, only VSV-vectored VLPs

have been evaluated in Gn pigs. It remains to be seen, if rNDV-vectored VLPs can induce immune responses as efficiently as VSV-vectored VLPs.

P particle vaccines

P particles have become increasingly appealing as vaccine alternatives to VLPs since their development by Tan and Jiang [81,101,112–113]. P particles are made by expression of the VP1 P domain with end-terminal cysteine residues in a prokaryotic expression vector [108]. P particles contain the P2 binding domain in the outer layer and P1 domain in the inner core [108]. Thus, P domain complexes retain the P2 HBGA binding domain [104] and have the same binding profile as the native capsid and VLPs [108].

Studies on immunogenicity and protective efficacy of P particles as vaccines have remained limited. VA387-derived P particles induced homologous, strain-specific HBGA binding blocking antibodies after IN inoculation in mice [101]. A study by Tamminen and colleagues [94] suggested that P particles were not as immunogenic as VLPs. However, Tan and Jiang [107] raised concerns that this study used P dimers, which are less immunogenic than P particles. A follow-up study demonstrated that P particles are indeed more immunogenic than P dimers and stimulate innate, cellular and humoral immune responses similar to VLPs in mice [105]. A recent study showed that an IN three-dose 100 μg GII.4/VA387-derived P particle vaccine regimen provided 47% protection against HuNoV gastroenteritis following cross-variant challenge with GII.4/2006b in Gn pigs [71]. An increased dose of P particles (250 μg) not only provided 60% protection against diarrhea but also against viral shedding [Kocher JF, Yuan L, Unpublished Data]. These protection rates closely mimic the protective efficacy conferred by the VLP vaccines previously reported in humans [76,79]. The three-dose P particle (100 μg) vaccine regimen also induces superior T-cell responses to the identical VLP regimen, including intestinal- and systemic-activated nonregulatory $CD4^+$ T cells, duodenal $CD8^+IFN\text{-}\gamma^+$ T cells and circulating Tregs following HuNoV challenge in Gn pigs [71]. Furthermore, the high-dose P particle vaccine regimen (250 μg) primed for increased IFN-γ-producing T cells and reduced Tregs in all tissues compared with the low-dose regimen. These data indicate that P particles have the potential to induce longer lasting immunity than natural HuNoV infection by potentially circumventing immune evasion by the virus, as was in the success of the human papillomavirus (HPV) VLP vaccine [114]. Further independent studies are necessary to compare the effectiveness and duration of P particle and VLP-induced protective immunity.

P particles are also capable of serving as a platform for expression of other viral antigens, including rotavirus [113], influenza virus [115] and hepatitis E virus (HEV) [116]. Immunogenicity studies of the P particles expressing these antigens have produced largely positive results. These compound vaccines increased cellular and humoral immune responses compared with free antigens in mice [113,116–117]. In summary, P particles are promising vaccine candidates due to their similar immunogenicity and protective efficacy as VLPs in Gn pigs. Additionally, the P particle platform provides a solid backbone for seasonal HuNoV vaccine development, if long-term protective immunity cannot be achieved.

Antinorovirus drugs

HuNoV antiviral development and clinical evaluation is a growing field. An excellent review on advances in HuNoV viral targets and antiviral development was recently published [118]. HuNoV typically presents as an acute, self-limiting infection that resolves within 72 h, so the current treatment relies on rehydration therapy for infected individuals. However, chronic shedders and immunocompromised patients can serve as potential reservoirs for emerging strains of HuNoV [54]. Effective antiviral drugs are needed to reduce HuNoV replication and transmission, especially in hosts that are incapable of clearing the viral infection. Recent antiviral studies have targeted the viral protease [119,120] or RdRp [121–123] or host proteins essential to the viral life cycle, such as deubiquitinase [124].

The HuNoV genome encodes several proteins that are rational targets for antiviral design, such as the protease and the RdRp. Inhibition of the protease prevents cleavage of the polyprotein into mature proteins for viral infection and particle assembly, while inhibition of the RdRp prevents replication of the viral genome. Several potential protease inhibitors have been reported [119,120]. One study reported reduced intestinal MNV titers in mice 3 days postinfection [119]. Novel antivirals targeting the viral RdRp focus primarily on nucleoside analogs, but development efforts have also focused on non-nucleoside analogs [123]. Both strategies have been effective in the inhibition of the viral polymerase [121–123]. Non-nucleoside inhibitors have also been effective against Norwalk virus replicon and MNV *in vitro* and *in vivo* [123]. On the other hand, targeting host factors in the viral life cycle is an alternative strategy that may limit viral evasion. Small molecule inhibitors targeting cellular deubiquitinase have been shown to reduce MNV replication and decrease levels of Norwalk virus RNA in the Norwalk virus replicon system *in vitro* and also have broad spectrum antiviral activity [124]. Research should continue to focus on the development of novel antivirals specific to HuNoVs. Existing preclinical antivirals, especially those already reported, require further validation and toxicity analysis.

Although there are no commercially available antivirals for HuNoVs, broad antiviral compounds have been successful against NoVs [125–127]. For example, nucleoside analogs ribavirin and favipiravir increased the number of mutations within the MNV genome, reduced the infectivity of isolated viral RNA and decreased the overall amount of infectious virus isolated from feces in MNV-infected mice [125]. Similarly, 2′-C-methylcytidine not only reduced viral shedding in infected animals but also prophylactically protected uninfected animals [127]. A more recent study reported that ribavirin resolved chronic HuNoV infection in two patients with common variable immunodeficiency, but did not have an effect in two other patients [106]. Other replication-dependent strategies have focused on compounds that inhibit the viral RdRp, such as suramin-related compounds [128]. Development of these compounds has previously been hampered by toxicity problems; modification of suramin reduced the toxicity but retained suramin's ability to potently inhibit both human and murine NoV RdRps [128]. The anti-NoV capabilities of these preexisting antiviral compounds increase the toolbox of healthcare professionals for treatment of persistently infected patients, while novel compounds are developed.

Other potential antiviral strategies have focused on re-stimulating the host immune system instead of targeting the virus itself, including interferons. In Gn pigs, animals treated with simvastatin were more prone to HuNoV diarrhea and virus shedding than non-simvastatin-fed pigs [45,129]. However, oral treatment of IFN-α following challenge abrogated simvastatin's effect on increasing virus shedding [129]. Similarly, a more recent study showed that IFNs α and β reduced systemic spread of MNV, but were unable to prevent persistent shedding and infection [89]. However, IFN-γ cleared persistent MNV infection with significant reductions in virus shedding 2 days postinfection [89]. These findings indicate that stimulating the host immune system of persistently infected yet immunocompetent patients may be effective for HuNoV treatment.

Conclusion

The slow progresses in the development of commercially available HuNoV vaccines and antivirals have so far inhibited our ability to control the spread of NoVs globally. This appears to be changing as several promising VLP candidates are in varying stages of clinical trials and P particles have emerged as viable vaccine alternatives also worthy of clinical trials. Current studies have demonstrated that both vaccine candidates are capable of expressing antigens from other viruses [90,97,116–117], which is promising to cost effectively reduce the economic impact of several pathogens. P particles have yet to be evaluated in human clinical trials; it will be interesting to see how they perform in humans. The newly reported vectored VLP vaccines are also promising and present their unique advantages.

Although these vaccine strategies are promising, the development of replicating vaccine strategies would be an important improvement over nonreplicating vaccines, especially the live oral attenuated vaccine for the pediatric population. Similarly, the importance of antiviral drugs for treatment of immunocompromised or chronic shedders cannot be overstated. Due to the potential emergence of strains from HuNoV quasispecies in persistently infected patients, the development of effective antiviral compounds could help to limit the emergence and spread of these strains.

Future perspective

Two studies have recently reported the development of *in vitro* B-cell and *in vivo* mouse model systems for HuNoVs [49,91]. These are the most promising reports of HuNoV infection and replication outside of the more expensive Gn pig and calf models [45,92] and human volunteers. However, these systems have not been independently validated nor consistently produced high levels of HuNoV replication. Research should remain focused on the development and refinement of cell culture and small animal models that result in robust replication of HuNoVs. The implications of a developed and consistent *in vitro* cell culture system or *in vivo* small animal model are obvious; these would afford the ability to produce the large amounts of virus for development of inactivated or attenuated HuNoV vaccines. It does remain to be seen how attenuated HuNoV strains as vaccines can protect against circulating homotypic, heterotypic and heterologous strains. Additionally, these model systems can improve the identification, screening and development of antivirals against

HuNoVs. Thus, one of the most important focuses for HuNoV research efforts remains on the development of consistent and cost-effective culture systems for the development of novel vaccines and antivirals.

Conventional and vectored VLPs and P particles derived from single HuNoV strains have been shown to be immunogenic against HuNoVs. Furthermore, limited studies and clinical trials have shown these vaccine strategies can be protective against HuNoV-induced diarrhea and, in some cases, infection. However, this single strain strategy often does not induce broad protection against multiple genotypes or ancient and emerging strains of HuNoV; the need for a broadly protective vaccine is of paramount importance. The recent strategy to build chimeric and multivalent vaccines [95] appear to be the most promising approach. By priming the host for multiple genotypes of HuNoV, these vaccines could be built around the circulating HuNoV strain while rationally targeting other circulating strains of HuNoVs. This strategy is similar to the current methodology for the influenza multivalent vaccines, another highly transmissible virus.

Similarly, HuNoV antiviral studies have been limited to preexisting antiviral drugs or by unideal screening methods due to the lack of a cell culture model and a reliance on surrogate viruses. Still, nucleoside analog-based antivirals, including ribavirin and favipiravir, have shown promising results in reducing replication and transmission of MNVs and HuNoVs *in vivo* and/or *in vitro*. The continued studies of antiviral compounds readily available should not be disregarded; rather, these compounds should be studied in clinical cases. Ribavirin has already been shown to have moderate clinical success [106]. Similarly, compounds that could stimulate the innate immune system also warrant investigation. A combination therapy of antiviral drugs and immunostimulatory drugs could function in tandem to clear the virus.

The development of an *in vitro* culture system will be particularly important for the development of antiviral drugs. First, the screening methods for antiviral drugs will improve as cell–virus–drug interactions will be able to be evaluated. Second, the full replication cycle of HuNoVs would be elucidated, detailing presently unknown viral–host interactions for rational drug targeting. Such targets could include compounds that impair, for example, RdRp–VPg interaction, viral protein translation, protease processing of the polyprotein, or viral assembly. Finally, a robust small animal infection model will allow evaluation of the actual efficacy of potential compounds and vaccine candidates for their effects on HuNoV infection before evaluation in the currently available but expensive Gn pig model of HuNoV infection and disease. It is reasonable to believe that HuNoV vaccines and new antiviral compounds could be developed in the relatively near future.

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EXECUTIVE SUMMARY

Background

- Human noroviruses (HuNoVs) are the leading cause of pediatric nonbacterial acute gastroenteritis following the implementation of rotavirus vaccines.
- HuNoVs result in >200,000 children deaths in developing nations each year.
- The elderly are more prone to severe outcomes following HuNoV infections.
- No efficient cell culture or small animal models are currently available, limiting vaccine development.

Norovirus epidemiology & economic burden: the need for vaccines & antivirals

- HuNoVs account for billions of dollars in economic burden worldwide each year.
- HuNoVs result in $180–$355 million in healthcare costs in the USA annually.
- An effective HuNoV vaccine could reduce economic burden by $2.1 billion over 4 years in the USA.
- Young children and the elderly would be the primary beneficiaries of a HuNoV vaccine.

Function of HuNoV proteins

- HuNoVs encode eight proteins across three open reading frames (ORFs).
- ORF1 encodes a nonstructural polyprotein, which contains a protease and a RNA-dependent RNA polymerase (RdRp).
- ORF2 encodes the major capsid protein, VP1, which is divided into two domains, the shell (S) and the protruding (P) domains.
- ORF3 encodes the minor structural protein, VP2, which increases capsid stability.

Genomic factors in HuNoV immunity

- Susceptibility to HuNoVs is related to histo-blood group antigens and secretor status.
- Histo-blood group antigens serve as a receptor for HuNoVs, though other receptors or coreceptors may exist.
- HuNoV infection does not result in lifelong immunity and reinfection can occur.

B cells in HuNoV immunity

- Antibodies are correlates of protection against HuNoVs.
- Antibodies reduce viral shedding and are critical for HuNoV clearance.
- Only IgG memory B cells persist for up to 6 months postinfection.

- Human and murine noroviruses (NoVs) have been shown to infect B cells *in vitro.*

T cells in HuNoV immunity

- $CD8^+$ T cells are critical for NoV clearance.
- $CD4^+$ T cells are a correlate of protection against MNVs.
- HuNoV infection induces a weak Th1 and strong Treg responses that may impact long-term immunity.

Virus-like particles vaccines

- Virus-like particles (VLPs) are nonreplicating vaccines that retain similar binding properties to the native virion.
- VLPs have been the most extensively studied HuNoV vaccine candidate. Several formulations are currently going through clinical trials.
- VLPs can be expressed at high yield in various eukaryotic expression systems, including baculovirus, yeast and plants.
- Single strain VLPs can provide homo-variant and cross-variant protection against HuNoV diarrhea.
- Chimeric and multivalent VLP formulations provide broad protection.

Vectored VLP vaccines

- Vector-based VLPs have been studied using vesicular stomatitis virus, Newcastle disease virus, adenovirus and Venezuelan equine encephalitis virus.
- Vectored VLPs can produce large amounts of VLPs within the host.
- Vesicular stomatitis virus and Newcastle disease virus VLPs induced stronger immune responses than conventional VLPs.
- Biosafety concerns and pre-existing host immunity may limit the development and deployment of vectored VLP vaccines.

P particle vaccines

- P particles only require a prokaryotic expression system, but can also be produced in yeast.
- P particles retain similar binding patterns as VLPs and HuNoVs even though they lack the S domain.
- Compared with VLPs, P particles have produced similar immune responses in mice and superior T-cell responses in Gn pigs.
- P particles provided cross-variant protection against HuNoV diarrhea in Gn pigs and can serve as a platform for expression antigens from multiple viruses.

Antinorovirus drugs

- Development of anti-HuNoV drug is important for reducing persistent infection.
- HuNoVs have numerous potential antiviral drug targets, including the viral protease and polymerase. Other targets include host factors necessary for viral replication.
- Nucleoside analogs have been shown to be effective against HuNoVs *in vitro* and *in vivo*.
- Ribavirin treatment was effective in virus clearance in 50% chronically infected patients with common variable immunodeficiency.
- Interferon treatments have been shown to be effective in reducing HuNoV replication in animal models. IFN-γ is a novel antiviral candidate against HuNoVs.

Conclusion

- Norovirus vaccine and antiviral development have been limited by the lack of small animal and cell culture systems.
- VLPs are the most promising vaccine candidate against NoVs and have several formulations in clinical trials. P particles have been effective in a large animal model, but have not been submitted to clinical trials.
- Antiviral drugs are necessary to clear persistently infected patients. These drugs would potentially reduce the emergence of novel strains of NoVs.

Future perspective

- A cell culture system and mouse model system have been recently reported for HuNoV. However, these systems have not been independently validated and do not result in robust viral replication.
- Refinement of these systems will be critical to the development of attenuated or inactivated vaccines and antiviral drugs.

Table 1

Host factors affecting norovirus susceptibility and immunity.

Factor	Type	Result	Ref.
Histo-blood group antigen type	O	More susceptible to Norwalk virus (GI.1) infection	[40]
Secretor status	Secretor (with functional *FUT2*)	More susceptible to GII.4 infection	[42,44]
	Nonsecretor (with *FUT2* inactivating mutations)	Associated with some non-GII.4 infection	[44]
Age	Young (<5 years of age)	Associated with more cases	[11]
	Elderly (>65 years of age)	Prone to more severe disease	[11]
Immunodeficiency	Natural and acquired immunodeficiency	Chronic infection	[45]

Table 2

Main characteristics of norovirus vaccine candidates.

Type	VP1 domain	HBGA binding pattern	Expression system	Size (nm)	As foreign antigen carrier	Animal studies	Clinical trials
VLPs	S and P	Same as native virion [82–85]	Eukaryotic: baculovirus [79], yeast [80], potato [89,90], tobacco [91], tomato [92]	27–38 nm	Yes: rotavirus [93]	Mice [86,87], rabbits [94], chimpanzees [95], Gn pigs [68,88]	Yes: several formulations [73–77]
Vector VLPs	S and P	Not determined	Viral: Vesicular stomatitis virus [96,97], Newcastle disease virus [98], adenovirus [99], Venezuelan equine encephalitis virus [100]	27–38 nm	Not determined	Mice [96–98]	No
P particles	P	Same as native virion [101]	Prokaryotic: *E. coli* [108], Eukaryotic: yeast [102]	~20 nm [102]	Yes: rotavirus [103–106], influenza [104–106], hepatitis E virus [105,107]	Mice [108], Gn pigs [68]	No

Gn: Gnotobiotic; HBGA: Histo-blood group antigen; VLP: Virus-like particle.