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REVIEW

Rotavirus vaccine efficacy: current status and areas for improvement

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ABSTRACT

The difference noted in Rotavirus vaccine efficiency between high and low income countries correlates with the lack of universal access to clean water and higher standards of hygiene. Overcoming these obstacles will require great investment and also time, therefore more effective vaccines should be developed to meet the needs of those who would benefit the most from them. Increasing our current knowledge of mucosal immunity, response to Rotavirus infection and its modulation by circadian rhythms could point at actionable pathways to improve vaccination efficacy, especially in the case of individuals affected by environmental enteropathy. Also, a better understanding and validation of Rotavirus entry factors as well as the systematic monitoring of dominant strains could assist in tailoring vaccines to individual's needs. Another aspect that could improve vaccine efficiency is targeting to M cells, for which new ligands could potentially be sought. Finally, alternative mucosal adjuvants and vaccine expression, storage and delivery systems could have a positive impact in the outcome of Rotavirus vaccination.

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Introduction

Vaccines are generally accepted as one of the most successful prophylactic interventions in history. Their development requires sustained efforts in research to improve efficacy and mitigate risks. Oral vaccines have great benefits as they can be administered more easily which can be critical especially during large, mass vaccination campaigns in developing nations. It is well established that environmental factors such as climate, poverty, nutrition and diseases take a great toll in the efficiency of vaccines. These circumstances can have a big impact on underprivileged populations, especially in developing nations. It can be argued that many healthcare solutions, most of which were originally developed to address the needs of higher income nations, are inadequate to the realities faced in developing countries. This can be inferred for instance from studies on the outcome of oral vaccines in such contrasting settings. In that regard, Environmental Enteropathy (EE) is believed to play a very significant, if not critical role.

Environmental enteropathy

EE is a subclinical condition frequently encountered in lower income nations and that can be attributed to a combination of factors, including repeated exposure to oral-faecal contamination, inadequate nutrition and poor overall sanitation. Ultimately EE may lead to small intestinal bacterial overgrowth and gut microbiome imbalances. EE also correlates with stunting of affected individuals, and a variety of other symptoms such as decrease in intestinal barrier function, reduced nutrient absorption capacity and increased gut permeability, which facilitates crossing of pathogens and subsequent activation of the immune system. It is likely that chronic gut inflammation contributes to growth abnormalities and stunting. Also, there are major histological modifications in the small intestine including shortening of villi.[1–3] Such changes may be reversed over time with improved nutrition and sanitary conditions[4] and lead to a lower nutrient absorption surface, which in itself reduces the benefit of interventions including supplements and vaccines. A critical time for EE onset is the transition from breastfeeding to complementary feeding as the risk for oral-faecal contamination is greatly augmented. The negative impact of EE can be mitigated with varying degrees of success with improved hygiene, nutritional supplements such as zinc and other micronutrients, omega-3-rich lipids, alanyl-glutamine, vitamins A and D, pro/prebiotics and also antibiotics.[1–3] For instance in an urban Bangladeshi slum, EE had a strong impact in oral polio and rotavirus immunization performance[5,6] but not in those parenterally administrated.[5] Gut microbiome, the administration of pro/prebiotics and their influence in vaccine efficacy has also received considerable attention with varying levels of success.[7]

Huda and colleagues analysed microbiome composition in stool samples from 48 Bangladeshi children immunized with oral polio virus (OPV), bacille Calmette-Guérin (BCG), tetanus toxoid (TT), and hepatitis B virus. They noted that *Actinobacteria* (especially *Bifidobacterium longum* ssp. *infantis*) were predominant and positively correlated with OPV, BCG and TT polyclonal T cell proliferation. The authors also found a negative correlation between presence of *Enterobacteriales, Pseudomonadales*, and *Clostridiales* and vaccine responses.[8] Another clinical

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study in India involving 551 infants analysed the influence of a probiotic and also zinc in the immunogenicity of oral Rotarix Rotavirus vaccine. After the normal course of two doses administered at 6 and 10 weeks of age with or without daily zinc (5 mg), probiotic (10^{10} *Lactobacillus rhamnosus* GG) or placebo, a gain in vaccine immunogenicity of 7.5% could be associated with the probiotic ($p = 0.066$) but not with zinc alone. This modest gain suggests that further studies with similar or other probiotic strains.[9] Also, Parker and colleagues used the same clinical trial setting to evaluate the incidence of 40 bacterial, viral and eukaryotic pathogens as well as the microbiome composition in 325 and 170 infants, respectively. The authors could not find a strong association between vaccine immunogenicity and the microbiome and noted also that colonization by *L. rhamnosus* GG had been passive rather than successful. Further, the pathogen burden in infants that failed to respond to vaccination was comparable to those that did.[10]

Overall, as no single solution can entirely overcome EE, a holistic approach is generally advised. Experts have postulated that reduced levels of EE may prove critical for increasing oral vaccine efficacy.[1–3]

Rotavirus disease biology and impact

It is estimated that worldwide, approximately 500,000 children under 5 years of age die annually as a consequence of diarrhea. Rotavirus gastroenteritis (RGE) is the leading cause of diarrheal death with a total estimate of 200,000 for all ages (146,000 among under 5 years old[11]). Rotaviruses were first identified by electron microscopy in intestinal biopsies of children diagnosed with acute gastroenteritis.[12] These viruses consist of non-enveloped, icosahedral particles comprising 11 segments of dsRNA encoding 6 structural (VP1-4, 6, 7) and 6 non-structural proteins (NSP1-6[13]). Cleavage of VP4 leads to VP5* involved in cell penetration, and VP8*, the head of the VP4 spike required for attachment and infection of intestinal cells.[14,15] Infection with naturally-occurring Rotavirus strains does not confer immunity but prevents strong diarrhea upon reinfection. Post reinfection, serotypic response is normally broader. Antibody responses are not limited to outer capsid proteins but include also VP2, VP6, NSP2, NSP4.[16] Neutralizing IgA specific to VP4 and VP7 prevents adsorption, uncoating and penetration, while VP6-specific IgA prevents intracellular viral replication during transcytosis across enterocytes.[13,14] Outer capsid proteins VP4 and VP7 are used in P (VP4 protease) and G (VP7 glycoprotein) serotyping and genotyping. Epidemiological studies indicate that Rotavirus diversity changes over the years within and between countries by point mutations (genetic drift) leading to antigenic changes, reassortment within and between animal strains, infections of other species as well as genetic rearrangements.[16] A review of the prevalence of Rotavirus strains in 100 reporting nations from 1996 to 2007 noted that strain diversity was associated with accumulation of point mutations driving antigenic drift, genome reassortment and zoonotic transmission. Five genotypes (G1-4, G9) accounted for 88.2% of all strains, with G1 declining from 2000 onwards and G3 re-appearing. Concurrently G9, G12 emerged and G8 strains increased in Africa, while G3-4 decreased.[17] It is interesting to note that in Africa, P[6] strains comprise 25% of VP4 genotypes while in Europe and North America these account for less than 4%.[18]

Rotavirus infection takes place via the apical tip of villi within the small intestine, with replication occurring primarily in differentiated epithelial cells, ultimately leading to cell death. This in turn leads to limited food digestion and release of fluids causing diarrhea.[13] Interaction with glycans is critical for early stages of Rotavirus infection. Histo-blood group antigens (HBGAs) expressed at the surface of red blood and epithelial cells can serve as non-sialylated glycan binding partners for Rotavirus entry via the VP8* protein within which a narrow cleft's wideness determines binding of cell-surface glycans. HBGA expression is genetically determined by the addition of monosaccharide to precursor disaccharides through the activity of *ABH* genes, FUT2 (*secretor* gene) and FUT3/4 (*Lewis* gene). Loss of function mutations in *FUT2* and *FUT3* lead to secretor- and Lewis-negative phenotype individuals which can determine Rotavirus specificity (e.g. VP8* of strain HAL1166 P[14] has a narrower cleft allowing binding to A-type HBGA) and A-type binding is also seen in P[9] and P[25] strains. Furthermore, P[11] strain is associated with high rates of infection in newborns from India with strain N155 recognizing type I, II precursor glycans (lacto-N-biose and N-acetyllactosamine, respectively) that become less prevalent with age.[15] Also, infection with the common VP4 P[8] genotype correlates with HBGA secretor status. For instance, loss of *FUT2* function leads to virtually no infection with P[8] strains in countries like Vietnam[19] and the USA,[20] but not in Tunisia where among 32 children infected, P[8] was present in both secretor and non-secretors that are positive for Lewis antigen.[21] Also, in Pakistan, a study comprising 181 infants that were seronegative before immunization with three courses of a G1P[8] vaccine strain indicated that 19% (10/54) of responders were non-secretors whereas secretors were 51% (20/39) type O and 30% (26/88) were types A, B or AB.[22] In addition, 18 P[6] infections (from a total of 27) were seen in predominantly Lewis-negative children (a frequent phenotype in African populations), while 27 Lewis-negative individuals were resistant to P[8] infection (0 in a total of 27 children). The same study noted that all 22 children in Nicaragua and 27 in Burkina Faso infected with P[8] strains were secretor Lewis positive.[23]

Rotavirus can also enter our bodies via M cells[24] that are located within follicle-associated epithelia (FAE). M cells possess structural and functional traits that allow transport of antigens to the adjacent mucosal-associated lymphoid tissue.[25] In the small intestine, M cells are concentrated around aggregates of lymphoid follicles called Peyer's Patches (PP). In infected individuals, production of mucus by the FAE is also reduced which facilitates uptake of pathogens by M cells and further processing by the underlying mucosal immune system.[26]

Overall, there is a great diversity in Rotavirus strains with a limited number of genotypes playing a dominant role. These have largely defined the makeup of Rotavirus vaccines to date. Host genetics also impact viral entry and as such vaccine

development could benefit from genotypic screening of regional and national populations. Similarly, as in the case of influenza, regular monitoring of prevalent viral strains could lead to better adjustments of vaccine to patients' needs, although in the case of Rotavirus this aspect has not been clearly established. Finally, taking into account the capacity of M cells for uptake of antigens, optimizing delivery to these could increase vaccine efficiency (see below).

Vaccine development and efficacy

Taking into account the mortality and morbidity caused by Rotavirus infection, several vaccines were developed over the years, most of which are live attenuated variants of naturally occurring strains. These vaccines were traditionally obtained after multiple rounds of passage in cell culture such as the pioneering bovine RIT4237 that demonstrated an overall efficacy of 50% in Phase III clinical trials but was never commercialized. Later-generation vaccines included a rhesus monkey-human tetravalent reassortant (RRV-TV, later licensed as RotaShield and soon after removed from market due to increased risk of intussusception, a rare, painful and potentially life-threatening bowel obstruction event), a human monovalent G1P[8] (RIX4414, commercialized as Rotarix by GSK; 2 doses from 6–24 weeks), and a pentavalent bovine-human reassortant (RV5, marketed by Merck as RotaTeq; 3 doses from 6–32 weeks[27]). Early studies have pointed at discrepancies in vaccine efficacy while comparing results from low and high child mortality settings.[28–32] Overall, a meta-analysis of Rotarix and RotaTeq performance (29 and 12 trials, respectively) involving 186,263 participants in low and high child mortality settings, indicates that in low mortality regions Rotarix prevents 86% and 85% of severe RGE cases in infants under 1 and 2 years of age, respectively while RotaTeq prevents 87% and 82% of severe RGE cases in infants under 1 and 2 years of age, respectively. In high child mortality regions, Rotarix prevents 63% and 42% of severe RGE cases in infants under 1 and 2 years of age, respectively, while RotaTeq prevents 57% and 41% of severe RGE cases in infants under 1 and 2 years of age, respectively. Both vaccines are regarded as safe with no significant difference in adverse events noted between vaccine and placebo groups. Intussusception cases were 58 in 97,246 children after Rotarix and 34 in 81,459 children following RotaTeq.[5,33–36] Another example is the Lanzhou lamb Rotavirus vaccine (LLR; genotype G10P[12]) which has been licensed in China since 2000 (one dose/year for 3 years, children 2 to 35 months old). Based on a study comprising 1,412 children immunized with at least one dose, the overall protection rate was found to be 35% (total population of 6,441 under 5 years old[37]). Rotavin-M1 (an attenuated G1P[8] strain developed and licensed in Vietnam) was regarded as safe and had comparable seroconversion to Rotarix in clinical trials.[38]

Other vaccines undergoing development, clinical trials and licensure include the naturally occurring reassortant monovalent human-bovine Rotavirus vaccine 116E (Rotavac, by Bharat Biotech International, strain G9P[11]). In a Phase III clinical trial the vaccine was administered at 6, 10 and 14 weeks of age (4,532 vaccine and 2,267 placebo) and based on observations up to 2 years of age, efficacy against severe RGE was 55.1%. There were 8 and 3 intussusception cases for vaccine and placebo groups, respectively (p = 0.7613).[39] Another vaccine under evaluation is a pentavalent bovine-human reassortant vaccine developed by the Serum Institute of India (BRV-PV, comprising serotypes G1, G2, G3, G4 and G9). A clinical trial taking place in Niger used a 3 dose regimen at 6, 10, 14 weeks of age for a cohort 1,780 and 1,728 infants in vaccine and placebo groups, respectively. The authors reported a 66.7% efficacy against severe RGE with no cases of intussusception.[40]

It is important to note that vaccine efficacy may also vary over the course of the year. Premkumar et al.[41] evaluated the efficacy of Rotarix and RotaTeq in the Americas depending on the level of exposure to Rotavirus season (defined by presence of Rotavirus in more than 10% of stool samples). This is the case from July to December in Bolivia, February to June in El Salvador, February to May in Guatemala, and January to April for Nicaragua and USA. Based on a cohort of 10,421 participants over the age of 6 months who had completed the full course of vaccination (1,690 cases and 8,731 controls), for infants under 12 months of age, vaccine efficacy was lower when born during Rotavirus season (72 vs. 84%) while for children above 12 months, overall there was no significant difference (76 vs. 78% during and outside Rotavirus season, respectively[41]).

In brief, there are currently two live-attenuated Rotavirus vaccines with global distribution, both of which show higher efficacy in developed nations. China, India and Vietnam also have vaccines licensed locally, and several more are being evaluated in clinical trials worldwide, including non-replicating vaccines. Further research in thermostable formulations will address the needs of developing nations which often lack the required cold chain capacity for storage and distribution of vaccines. In the Americas, vaccine efficacy appears to decrease during Rotavirus season. If these observations translate into the rest of the world, adjustments to dosing could potentially improve outcomes of immunization. Live attenuated vaccines are not without risk, as illustrated by RotaShield and mitigation of adverse events through improved dosing regimens and safer strains are therefore important considerations (see below).

Vaccination risks and benefits

In the aftermath of the voluntary withdrawal of RotaShield from the market in 1999 due to increased risk of intussusception, the safety and benefits of Rotavirus vaccines have been the subject of substantial scrutiny and comment. A USA-based report evaluated the occurrence of intussusception in 19 states in infants aged 1 to 12 months and noted that 429 had developed the condition, 74 of which had also been vaccinated with RotaShield. The incidence was greater 3–-14 days after vaccination, especially after the first dose (43 accounts; 9 after second and 1 after third) with an estimated 28% increase in cases of intussusception if the vaccination program were fully implemented (1 in 4,670 to 9,474 infants vaccinated). Overall the study showed strong temporal and specific association between vaccine administration and

intussusception and concluded that vaccines with better safety profile are required.[42] Another study evaluated the impact of age in incidence of intussusception after RotaShield administration and noted that infants over 90 days old at first dose represent 80% of all cases (despite having received solely 38% of first doses). Overall, vaccination schedules could be completed by 60 days of age in 2 doses so as to reduce intussusception incidence.[43] This strategy was tested with RRV-TV in Ghana (despite being withdrawn from European and USA markets) and based on data from 500 infants in vaccine and 498 in placebo groups, efficacy was 63.1% against RGE of any severity for serotypes present in vaccine and 60.7% for all serotypes. No cases of intussusception were reported[44]. A post-licensing evaluation of intussusception risk in the USA comprised 1,277,556 doses for RotaTeq (of which 507,874 were first doses) and 103,098 doses for Rotarix (of which 53,638 were first doses). The authors performed two analyses and for RotaTeq the primary analysis indicated an association between vaccination and intussusception (highest at 3–7 days after first dose; 1.1 excess cases/100,000 infants receiving the first dose for the 7-day risk window and 1.5 excess cases/100,000 infants receiving the first dose for the 21-day risk window); the secondary analysis identified an attributable risk of 1.2 excess cases/100,000 infants receiving the first dose for the 21-day risk window (no risk attributable to doses 2 and 3). The limited number of Rotarix doses did not allow for precise estimation of intussusception risks therefore preventing a direct comparison with RotaTeq. The secondary analysis indicated a significant attributable risk after the second dose of 7.3 excess cases/100,000 infants for the 21-day risk window[34] It will be important to continue monitoring risks associated with Rotavirus vaccines in low income nations as data from high income settings may not directly compare.[45] Ironically, although efficacy of Rotavirus vaccination is lower in developing nations, it is also in such regions that the benefits are of greater magnitude due to higher burden of disease.[33,46–49] Taking into consideration the disease burden and associated costs of hospitalization as well as the mortality of Rotavirus gastroenteritis, the impact of vaccination is predicted to be very significant.[47] It is estimated that in GAVI-eligible countries, vaccination could prevent 2.46 million child deaths and 83 million disability-adjusted life years.[46] According to Troeger et al., from 2005 to 2015 there was a decrease in mortality of 43.6% among children under 5 years of age which was probably due in part to vaccine introduction.[11]

It is possible that mass introduction of vaccines could lead to emergence of escape mutants. In that regard Dóró et al.[50] looked into the prevalence of Rotavirus strains over the course of 6 years after introduction of Rotarix and RotaTeq (2007–2012). Strain frequencies were similar to before vaccination campaigns, the dominant ones being G1P[8], G2P[4], G3P[8], G9P[8], G4P[8] and G12P[8]. Numerous countries reported an increased frequency of strains that emerged in 1990s (i.e. G9P[8] and G12P[8]) and no evidence for global emergence of new ones. However, studies from Brazil[51] and Nicaragua[52] suggest genetic reassortment between vaccine and wildtype (WT) strains for Rotarix and RotaTeq, respectively, which probably had no influence in vaccine efficacy.[52] There is also a higher frequency of heterotypic G2P[4] in countries after Rotarix use but this was also observed in RotaTeq-adopting countries within the same regions. Generally, natural strains variation over the years could be mistaken for genetic drift caused by introduction of vaccines.[17] Further monitoring will be required to assess strain evolution especially if it leads to lower vaccine efficacy.[50]

Overall, Rotavirus vaccination clearly outweighs the risks of adverse events such as intussusception across geographies and demographics. Simple strategies including avoiding immunization at ages of peak natural occurrence can significantly reduce intussusception incidence. Further, there is no compelling evidence for selection pressure on naturally occurring strains due to mass introduction of Rotavirus vaccines but continuous monitoring is advised.

Areas for improvement and further research

In addition to the areas of intervention discussed above (e.g. sanitation, environmental enteropathy, nutritional status, maternal antibodies), there are several established and emerging areas that could allow for improving the efficacy of oral Rotavirus vaccines in developing nations. These include the optimization of delivery, mucosal adjuvants, finding actionable targets through systems vaccinology approaches, and chronobiology of mucosal vaccine delivery.

Optimizing vaccine delivery

Mucosal surfaces of human adults comprise about 400 m^2 (200-fold higher than skin area). Therefore they present ample opportunities for both pathogen entry (70% of all infections take place via this route) and also immunization. An ideal mucosal vaccine should lead to appropriate adaptive response without excessive inflammation, tissue damage or other side effects arising from stimulation of wrong target cells such as mucosal epithelial cells for e.g. production of proinflammatory cytokines. Antigen uptake and presentation in intestinal and lung mucosal tissue relies on dendritic cells (DCs) and M cells. The latter comprise a basolateral pocket that facilitates transcytosis of antigens, such as viruses and bacteria, and contact with underlying lymphoid tissue. Antigens are delivered to follicular B cells or mucosal DCs that present such cargo to T cells or transit to mesenteric lymph nodes.[53] The fact that M cells are sparse (~1 in 10 million intestinal epithelial cells) may reflect a mechanism of self-protection against food allergy and inflammatory disease.[54] Mice studies show that M cells differentiation is a highly regulated process.[55]

Although increasing M cell number has been achieved with modulators of their proliferation (e.g. RANKL[56]), targeting of M cells with specific ligands may be a safer approach. An ideal M cell marker would be membrane-bound (to favour internalization of tagged cargo) and also conserved among different humans and preclinical animal models for enabling efficient translation of results. However, finding such universal M cell markers has proven difficult.[57] Research in this field includes work on Lewis A antigen[58] and GP2.[59–61] Several groups have searched for M cell-specific targeting

ligands. Kim et al.[62] screened a human M cell model co-culture by phage display with a peptide library. One peptide (Co1; SFHQLPARSPLP) facilitated transcytosis of GFP-Co1 fusions to M cells in mouse intestine and that also led to higher anti-GFP serum IgG and faecal IgA upon oral immunization.[62] Similar data was reported for Co1 fusions to Dengue virus envelope domain EDIII protein.[63] Also, mice that were orally immunized with GPF-OmpH β1α1 peptide fusions (AKIAIVNVSRIFQQLPESET) led to higher IgG and faecal IgA responses towards GFP as compared to GFP alone or GFP-Co1 fusions.[64]

Mucosal vaccines can become too diluted in fluids and miss target due to bulk flow while being degraded by proteases so it is critical to appropriately design them to retain ideal biophysical properties. Hydrophilic and un-charged surfaces favour mucus penetration while adhesion to mucus is promoted by hydrophobicity/positive charges (e.g. chitosan, cellulose derivatives). Polyethyleneglycol can also favor adhesion to mucus or diffusion, when ≥10 kDa or ≤2 kDa, respectively.[53] Another example of a peptide found by phage display mediating M cell entry is that of CKS9 (CKSTHPLSC), which was conjugated to swine dysentery bacterial antigen BmpB and loaded into poly (lactic-co-glycolic acid), PLGA particles coated with water-soluble chitosan. Oral immunization of mice with these particles led to increased faecal sIgA and IgG responses.[65] Importantly, PLGA is frequently used as a drug delivery system and regarded as safe.[66] Singh *et al.*[67] described an ileum-targeted protein delivery system based on commonly used and safe hydroxypropyl methylcellulose phthalate in thiolated form (T-HPMCP) for delivery at pH≥.4, while favouring mucoadhesion and as such slower release of the payload. Upon oral immunization of mice, M-BmpB antigen encapsulated in particles harbouring CKS9 showed ~4.7-fold higher IgA and IgG content in faeces and serum, respectively, as compared to M-BmpB alone.[67] Human M cell are frequently studied as co-cultures of Caco-2 and Raji cell lines. Recent work showed that human intestinal crypts grown as monolayers can differentiate into M cells upon addition of RANKL, leading to 18-fold increase in microparticle uptake.[68] The latter and other novel systems could be of interest to identify new markers and improve the current understanding of M cell biology.

Enzymatic degradation and clearance via mucus secretion and bulk movement limit efficiency of oral vaccine delivery to some extent. Some forms of transgenic plant-expressed antigens can protect from gut digestion without inducing tolerance if properly dosed.[69] For instance, transgenic rice expressing Cholera toxin B ('MucoRice-CTB') has shown potential for expression and long-time storage of vaccine antigens in seeds within a storage organelle (protein body) where it remains protected from digestion therefore retaining immunogenicity. After ingestion of rice powder (50 mg/course, 75 μg CTB, total of 6 doses every 2 weeks/mouse), uptake via M cells induces IgG (serum) and sIgA (faeces) while oral administration of similar amounts of recombinant purified CTB did not or barely elicit IgA (there was some incidence of diarrhoea in latter but none in the former[70]). MucoRice-CTB also provides cross-protection to enterotoxigenic *E. coli* with lower levels of IgG, faecal sIgA than toward CTB.[71] The technology is currently being evaluated in clinical trials.[72]

Oral immunization in regions affected by environmental enteropathy leads to lower vaccine efficacy. Alternative forms of administration such as microneedle transdermal patches could therefore be of interest to bypass this constraint. These target dense network of antigen-presenting cells (APCs) in dermis and epidermis (compared to skeletal muscle) and provide overall similar or increased immunogenicity to intramuscular delivery while showing high stability and dose-sparing capabilities. Microneedles are generally regarded as safe in clinical trails,[73] can be simply and painlessly applied in a single dose without requiring special training, vaccine reconstitution and sharp waste. Taking into account that they can be mass-produced for less than US$0.10 per unit, microneedle patches could be economically appealing in low income areas.[74] These can be solid metallic, silicon or polymeric coated with dried vaccine or self-dissolving (e.g. vaccine encapsulated in water-soluble materials that release antigen/adjuvant in skin). Vaccines can be made available in dry formulations which may comprise stabilizers and adjuvants, thus reducing cold chain needs. These have been applied in preclinical and clinical studies for a variety of pathogen sources such as live attenuated viruses, inactivated viruses, viral subunits, VLPs, bacterial antigens and also DNA vaccine.[73,74] For instance Moon et al.[75] compared single dose stainless steel microneedle patch and intramuscular injection for the delivery of unadjuvanted human CDC-9 inactivated Rotavirus vaccine in mice. They found that the IgG level induced by a 0.5 μg dose delivered with microneedles was at least as efficient at the 5 μg dose given via intramuscular route 28 days after injection. Also, spleen analysis indicated that both delivery methods seemed equally efficient in inducing a recall response.[75] Another study tested a hollow microneedle injection device to deliver three 5 μg doses of CDC-9 G1P[8] vaccine without adjuvant and compared it with equal intramuscular (IM) injections adjuvanted with aluminium hydroxide in piglets (days 0, 10 and 21). Following challenge with human Wa G1P[8] Rotavirus (day 28), the authors found no signs of reactogenicity at the site of injection and at day 21 IgA and IgG serum mean titres were 19- and 20-fold higher after IM administration compared with microneedle, with no significant differences at day 28 (but still higher for IM). Evaluation of viral shedding and diarrhoea after challenge indicated stronger protection for piglets immunized with microneedle than IM.[76]

Another approach to overcome the barriers that EE pose to classical oral vaccine delivery is sublingual administration. Advantages over dermal vaccines include absence of keratinized surface cells that prevent simpler access to APCs (buccal, sublingual routes lead to antigen uptake in 30–60 min). Differences in saliva composition, pH and flow rate are potential obstacles to buccal vaccine delivery. Examples include live attenuated viruses and inactivated vaccines for influenza, adenoviral vectors to deliver antigens in pre-clinical models (e.g. influenza HA, HIV, Ebola) and bacterial vaccines such as *B. subtilis*-expressing toxins. Inactivated vaccines include non-replicating formulations that require adjuvants such as CpG and detoxified CT and LT for

proper mucosal response (e.g. HPV, RSV, HIV component proteins worked in pre-clinical assays[77]).

Intranasal vaccination also shows promise provided that vector and adjuvants are properly sourced.[78,79] The technology is currently approved for live attenuated influenza vaccine.[80] It is therefore possible that this route of administration could be used to circumvent the limitations of oral Rotavirus immunizations. Interestingly, simian and porcine Rotavirus NSP4 have shown adjuvant properties in mice when co-administered with model antigens, increasing both serum IgG and faecal IgA responses.[81] In addition, intranasal (and intramuscular) delivery of recombinant human Rotavirus VP6 combined with Norovirus virus-like particles led to a reduction of at least 65% in Rotavirus shedding in faeces upon Rotavirus challenge in mice.[82]

Live cultures of lactic acid bacteria are also used for mucosal vaccine delivery as they are quite resistant to gastric acid but further encapsulation protects surface-displayed antigens from adverse conditions. Also, different strains have distinct adjuvant and adhesive abilities, can persist in the gut for varying amounts of time and can lead to the expression of diverse cytokines.[83] Examples include oral and intranasal delivery of SARS coronavirus spike protein displayed by *Lactobacillus casei* in mice leading to specific serum IgG as well as intestinal and lung IgA responses with *in vitro* neutralization activity.[79] Also, *Lactococcus lactis* expressing HPV16 E7 antigen with or without co-administration of strain expressing IL-12 were used in intranasal immunization of mice. Following lethal challenge with tumour cell line TC-1, 50% of mice treated with E7/IL-12 were tumour-free after 100 days while the remaining mice had significantly smaller tumour sizes (~1 cm^3) than controls immunized with *L. lactis* alone (all of which perished after 35 days; median tumour size ~ 6 cm^3).[78]

In summary, M cell targeting has received considerable attention for increasing mucosal vaccine efficiency. Although their low numbers can be raised, the risk of adverse reactions led several groups to discover naturally occurring or randomly selected M cell-specific targeting peptides. When tethered to antigens these moieties lead to higher immune responses in mucosa and serum. In addition, encapsulation of antigens in nanoparticles allows for protection of cargo from digestive tract enzymes, mucoadhesion and pH-dependent release at different locations of the gut, such as M cell-enriched ileum. Alternative immunization methods comprise e.g. transgenic plants that may not only allow for vaccine antigen expression and delivery but also long term storage. Also, intradermal, buccal/sublingual and intranasal delivery could offer solutions to overcome the barriers of EE.

Mucosal adjuvants

Oral administration of vaccines is challenging in part due to hyporesponsiveness and tolerance issues. Adjuvants act by enhancing and modulating immune response therefore allowing for lower and less frequent dosing and as such antigen saving, which can prove critically important in case of pandemics. They also hold promise for enhancing responses in children, immunosenescent and immunocompromised patients. Adjuvants stimulate the innate immune system by acting on DCs, macrophages and neutrophils, and also on the adaptive immune system. As discussed above, encapsulation in biodegradable particulate delivery systems and mucosal adjuvants is a promising approach for protection from digestive enzymes.[84] Live attenuated vaccines can harbour multiple antigens that also serve as adjuvants. Inactivated vaccines generally have lower efficacy as these components may lack ideal function. Subunit vaccines also show poor immunogenicity and therefore need an adjuvant to elicit satisfactory responses.[53] For instance, encouraging results have been reported in preclinical studies by grafting epitopes onto appropriate scaffolds (e.g. HIV1 gp41[85,86]). Further, immunization with structurally similar regions of different pathogens e.g. *Candida albicans* adhesin Als3 conferred protection against *Staphylococcus aureus* via its adhesin ClfA.[87] Similar observations have been made with targeted substitutions within *N. meningitidis* fHBP (a component of Bexesero vaccine[88]). These results emphasize the need for discovery of suitable mucosal adjuvants such as bacterial toxins that can induce strong local and systemic immune responses. An example that reached commercialization was an inactivated intranasal virosomal-subunit influenza vaccine supplemented with *E. coli* ADP-ribosylating heat-labile toxin (LT) as a mucosal adjuvant. Although prelicensure trials including 1218 individuals over 4 winters showed no serious side effects, following introduction of the vaccine 46 cases of Bell's Palsy were recorded over the course of 7 months. This represented an estimated 19-fold relative risk increase, leading to the vaccine being withdrawn from the market.[89] Although LT and also cholera toxin can be detoxified, their use has been limited to animal models and clinical trials.[90–92] Detoxified versions of bacterial toxins are normally less potent adjuvants than the original molecule.[93] A phase I clinical trial with an intranasal influenza vaccine comprising a mutant detoxified form of LT toxin (LTK63) was well tolerated with the adjuvant dosed at 3, 10 and 30 μg within a cohort of 70 volunteers.[94] However, in phase I clinical trials with intranasal HIV and tuberculosis vaccines adjuvanted LTK63 at a dose of 30 μg, two healthy volunteers showed symptoms of transient Bell's Palsy. These data indicated that nasal delivery of the toxin is not advisable.[95] In that regard, the LT double mutant dmLT (R192G/L211A) was used as an adjuvant in a clinical trial for an oral enterotoxigenic E. coli vaccine and shown to be well tolerated and effective in increasing immune responses among a cohort of 129 Swedish volunteers.[96]

Certain cytokines also show potential as mucosal adjuvants. Cholera toxin (CT) can increase levels of IL-1, IL-6 and IL-8 in mucosal epithelial cells. Therefore IL-1α and IL-1β were tested as adjuvants in intranasal immunization of mice with ovalbumin and tetanus toxoid and shown to increase IgG and IgA in vaginal lavages in a manner comparable or superior to CT.[97] Similarly, Kayamuro et al.[98] tested 26 interleukins as adjuvants in intranasal immunizations of mice with influenza HA and noted that IL-1 family members (IL-1α, IL-1β, IL-18, IL-33) were effective in increasing IgG and IgA levels in serum and saliva, nasal wash, faecal extract and vaginal wash, respectively. The authors also found that upon viral challenge, all mice immunized with HA and cytokine

adjuvants were alive after 7 days while within the same time frame, 86% of those receiving HA only had perished, and none mock vaccinated with PBS were alive. There were also no signs of severe inflammation or tissue damage in the nasal cavities.[98] It is interesting to note that there is some evidence for a role of glutamine in favouring intestinal IgA production.[99]

Several studies describe the induction of mucosal immunity after parenteral administration of adjuvants. Heine et al.-[100] used *E. coli* double mutant LT R192G/L211A (dmLT) for improving immune response to *Shigella* antigens IpaB and IpaD delivered intradermally using microneedles. Mice were immunized 3 times at 2 week intervals leading to quick infiltration of neutrophils, macrophages, dendritic and Langerhans cells, as well as $CD4^+$ and $CD8^+$ T cells. IgG levels in serum were elevated but contrary to observations made with control intranasal immunizations, no IgA was detected in serum or stools. Intranasal delivery also led to antibody IgG and IgA secreting cells in lung, spleen and bone marrow while intradermal injection only led to IgG secreting in spleen and bone marrow. Interestingly, intradermal vaccination provided up to 70% and 50% protection against lethal pulmonary challenge with *S. flexneri* and *S. sonnei*, respectively (50% and 20% without dmLT adjuvant) while intranasal vaccine delivery provided 100% protection. Only antigen-specific IgG was present in lung mucosal fluid after intradermal immunization while both IgG and IgA were detected after intranasal delivery.[100] Frederick et al.[101] compared the effect of CpG and dmLT in combination with MHC class II $CD4^+$ T cell peptide antigen 2W1S after intramuscular and intradermal ear immunization in mice. dmLT was superior to CpG for expanding and maintaining antigen-specific $CD4^+$ T cells in lymph nodes and spleen and at inducing intestinal homing integrin α4β7 in spleen and mesenteric lymph nodes. Small and large intestine lamina propria also had greater number of antigen-specific $CD4^+$ T cells. Intramuscular and intradermal immunization in the flank gave similar results indicating that adjuvant and not route of administration determines T cell migration to intestine tissue. While CpG leads to a Th1 $CD4^+$ T cell bias, dmLT results in both Th1 and Th17 responses.[101]

In brief, adjuvants play a major role in stimulating immune responses, especially with inactivated or subunit vaccines. Although several proven single component and combination of adjuvants are available for parenteral immunization, mucosal adjuvants are harder to develop. Following toxicity problems in the clinic with *E. coli* LT, detoxified versions of LT and CT, as well as cytokines, are being explored in clinical trials. Intradermal immunization adjuvanted with dmLT seems to trigger IgG and antigen-specific $CD4^+$ T cells in intestines but not IgA (while the latter is present after intranasal delivery in serum and stool samples).

Genetics of vaccine response and systems vaccinology

Genetic makeup, environment, age, gender, microbiome and body-mass index, all exert great influence in the outcome of immunizations.[102,103] To better understand the diversity of an individual's response to vaccines and therefore predict their outcome, several groups have pursued genetics and systems biology/vaccinology approaches, looking essentially at the outcome of parenteral immunization. Other studies have integrated information gathered from genomic variation, gene expression, cytokines and chemokines analysis, multiparameter flow cytometry, metabolome and computational modelling.

For instance, a study in The Gambia with 48 monozygous and 159 dizygous infant twin pairs living together looked into environmental vs. genetic regulation of immune response to BCG, oral polio, HepB, diphtheria, pertussis, tetanus at 5 months. All antibody responses were heritable, particularly in the case of HepB and polio vaccines, and genetic factors, specially non-HLA genes play important role modulating vaccine response in infants.[104] Another twin study analysed 78 monozygous and 27 dizygous healthy pairs (8–82 years old) for 204 different immune parameters comprising cell population frequencies, cytokine responses and serum proteins. Both innate and adaptive cell population frequencies were influenced mostly by non-heritable parameters (61% of all populations) but others were under very strong influence of heritable factors. The article also reports that the 51 serum proteins measured showed great variation in heritability. In addition the authors looked at variation in heritability of traits between young and older MZ twin cohorts (<20, 13 median and >60, 72 median years old, respectively) and noted that in several cases, there was a decrease in correlation with age, likely reflecting different environmental exposures and possibly also epigenetic changes. Further, analysis of antibody responses to seasonal influenza vaccine (2009, 2010, 2011) indicates influence from mostly non-heritable components. Overall, heritability of immune traits tends to decrease with age, likely reflecting the effect of exposure to environment.[105] These and other studies indicate genetic influences to immune responses associated with both coding and non-coding regions of the genome and that are under strong influence of environmental factors.[106–109]

Querec et al.[110] also used systems vaccinology methods to identify markers that predict responses to yellow fever vaccine YF-17D. They evaluated 15 healthy volunteers (in 1st year) and 10 (in 2nd year) at days 0, 1, 3, 7, 21 and noted that 65 genes such as CXCL10 and IL-1α were modulated in both trials (associated with immunological responses and cell motility). Peak gene expression were observed at 7 days, with the largest category involved in antiviral and interferon responses. Interestingly, transcript levels of TNFRSF17/BLys-BAFF at day 7 were predictive of antibody responses for both trials.[110] Another example of systems vaccinology came from the analysis of signatures for live attenuated (LAIV, n = 28) and trivalent inactivated influenza vaccines (TIV, n = 28). Data showed poor responses for LAIV at day 28. CXCL10 was induced in plasma samples from TIV and microarray data from PBMCs of the 56 individuals at days 0, 3, 7 shows 1,445 genes differentially modulated in both vaccines (including common inflammatory and antimicrobial genes for TIV, LAIV, and also interferon genes modulated for LAIV, likely due to virus replication). Overall, there were distinct signatures for LAIV and TIV with the latter vaccine also showing upregulation of TNFRSF17. Antibody responses were

predicted by kinase CaMKIV which is involved in T cell development, inflammatory response and maintenance of hematopoietic stem cells. At day 3, this parameter negatively correlates with antibody response on day 28 and immunizing KO CaMKIV and control WT mice with TIV led to 3 to 6.5-fold higher response on day 7 for KO.[111] Similar systems vaccinology analyses were published by Tsang et al.[112] for influenza vaccine and Li et al.[113] for meningococcus vaccines. Nakaya et al.[114] and Sobolev et al.[115] also used multifactorial analysis to study the impact of adjuvants in influenza vaccines. Metabolomics has also been recently employed.[116]

Overall, despite the growing interest in systems vaccinology there is a lack of common genetic markers that can predict immune responses, with the vaccine type determining broad transcriptional outputs (e.g. bacterial polysaccharide vs. inactivated viral vaccine). Also, adjuvants can mitigate variation in response and magnitude associated with children and elderly subjects. In addition, there is a need for further integrative 'omics' studies to fill the current gaps in our understanding in mucosal adjuvants, especially in young children. In theory, rational modulation of positive or negative regulators of vaccine response could potentially improve the efficacy of vaccination campaigns.

Circadian rhythms and their influence in immune responses

An estimated 10% of the genome is under circadian control, which is regulated by light patterns. Food intake is another powerful cue. The circadian clock is an important gatekeeper for reducing immunity-associated costs and for increasing overall fitness, as mediated by external cues and internal oscillators of immune cells. Nearly all branches of innate and adaptive immune response show circadian oscillations.[117–119] CLOCK:BMAL1 activates transcription by binding to E-boxes of PER, CRY and REV-ERB which after a delay enter the nucleus and repress CLOCK:BMAL1 activity (and as such their own transcription). RORα activates transcription of *Bmal1* by competing for the same binding sites as repressor REV-ERBα, β. A third loop includes transcriptional activator DBP and repressor NFIL3 that regulate expression of D-box-controlled genes such as PER. Ultimately these transcriptional oscillations in key regulators lead to circadian cues for cellular process over 24h cycles. Peripheral clocks are orchestrated by central clock and use essentially the same components, in virtually all cells. Some diseases show circadian timing (e.g. in RA, symptoms are exacerbated in early morning, which correlates with high TNF and IL-6 serum levels) and to address this matter, chronopharmacology aims at optimizing drug administration for peak efficacy and minimal side effects.[117–119]

For gaining a better understanding of circadian regulation of global gene expression, Zhang et al.[120] used transcriptomics of 12 organs from mice (adrenal gland, aorta, brainstem, brown fat, cerebellum, heart, hypothalamus, kidney, liver, lung, skeletal muscle, and white fat) to analyse circadian gene expression by microarray and RNAseq. They found that 43% of protein-coding genes oscillated in at least one organ (only 10 transcripts oscillated in all, which included core clock genes). The authors also noted a 'rush hour' for transcription of circadian genes at times preceding dawn and dusk and that circadian genes cluster physically within the genome. Importantly, many gene products physically modulated by drugs show circadian patterns (119 of WHO's essential medicines modulate circadian genes; these include 56 of US's top 100 selling drugs, nearly half of which have half-lives < 6h). These findings emphasize the importance of dosing at appropriate times while decreasing side effects to a minimum.[120] Circadian regulation of gene expression was also noted in mice intestine, including for pattern-recognition receptors TLR2, −3, 4, −5, 9.[121]

Circadian rhythms influence critical components of innate and adaptive immunity. An example of this is TLR9, a pattern-recognition receptor for bacterial and viral DNA CpG. Following mice vaccination with ovalbumin adjuvanted with CpG at times of higher TLR9 expression, the adaptive response is stronger. Also disease severity in a sepsis mouse model correlated with TLR9 levels.[122] Circadian rhythms also affect viral replication. For instance, Edgar et al.[123] addressed the effect of circadian rhythms in herpes and influenza virus using murine strains to infect WT and *Bmal1* KO mice and cell lines. WT mice infected with recombinant murid herpesvirus 4 (MuHV-4) at onset of resting phase had 10-fold greater replication than those infected before active phase. In contrast *Bmal1* KO showed high levels of viral replication at any time. Similar data were obtained using herpes simplex virus 1, indicating that the effect was not strain-specific.[123] Circadian rhythms could therefore modulate response to vaccines, regardless of the route of immunization. Sleep deprivation is known to disrupt circadian rhythms and correlates with lower vaccine responses to hepatitis A[124] and hepatitis B.[125] However, Karabay et al. did not notice a difference in hepatitis B responses between volunteers vaccinated in the morning or afternoon.[126] Also, Long et al.[127] analysed the impact of time of day (9–11 am vs. 3–5 pm) in influenza vaccine efficacy in a cohort of 276 > 65 year old UK citizens showing no immune disorders or infections (2011–13; 141 morning and 135 afternoon vaccinations). Evaluation of antibody titres, cytokine and steroid hormone levels in serum took place before and 1 month after vaccination. Strain A/H1N1 showed higher response to morning vaccine while A/H3N2 and B strains did not (HAI assay), with no influence of cytokine and steroid levels.[127] Another report analysed the impact of sample collection and immunization timing in elderly (n = 80, above 65 years old) and younger individuals (n = 59, ages within 30–40) with blood samples collected before (day 0) and 7 and 14 (or 28) days after vaccination. While there was no evidence for effect of timing of immunization, data indicates a significant increase in IgG and IgM titres at 7 days for the older group (but not younger) when samples were collected in the afternoon. This suggests that time of sample collection can affect interpretation of vaccination outcomes.[128]

In brief, circadian rhythms play a critical role in modulating the expression of genomes, including genes associated with therapeutic intervention. Innate and adaptive immune systems are not exceptions and intestinal tissues are also

under such regulation. Evidence also suggests that circadian rhythms can play a role in virus life cycle. Therefore, timing of immunization could potentially be important. Further studies are required, especially in the field of mucosal vaccination.

Discussion

The difference in Rotavirus vaccine efficacy noted between high and low-income countries indicates that novel, more appropriate and effective vaccines should be developed to address the specific requirements of the populations that need them the most. Currently, there are knowledge gaps in mucosal immunity and its response to Rotavirus infection. Increasing our understanding in this area, especially among young children, would likely provide critical insights. There is also little information on the role of circadian rhythms in vaccine response and more studies, specially addressing mucosal immunity, are needed. It is however known that in pre-clinical models, virus infection is affected and also that for instance the expression of critical pattern-recognition receptors associated with responses to pathogen such as TLRs (including in intestinal tissue) depends on circadian rhythms.[121,123]

In order to address these current gaps in knowledge, performing an integrative systems vaccinology 'omics' analysis[102] of intestinal mucosal immunity with healthy humans of various ages and ethnic/genetic backgrounds, before and after (Rotavirus) vaccination, could prove informative. This would comprise also an evaluation of circadian patterns of expression, including genes associated with pathogen entry. The study could then be extended to individuals affected by different levels of entheropathy from matching age/ethnic/genetic backgrounds. Analysing outputs could provide new venues for intervention to compensate for defects in critical pathways relating to e.g. pathogen entry and immune responses that are affected in sick individuals. Simple solutions such as using pertinent adjuvants, as well as timely provision of supplements and possibly also sourcing locally available food and remedies. In addition, circadian gene expression patterns would potentially give indications on whether vaccine administration at specific times of day could contribute to a higher efficacy.

Growing evidence suggests that more studies are required to further validate and identify critical Rotavirus entry factors in individuals with different genetic backgrounds.[15,129] Exploring and potentially improving emerging cell culture systems that are better mimics of gut tissue[68] could prove very useful as they would assist in identifying and evaluating specific Rotavirus entry factors in cell cultures representing genetically diverse individuals. These specific entry factors could potentially be used as markers to easily probe children's genetic backgrounds with low cost kits as preliminary stages to mass vaccination campaigns, following which tailored vaccines would be administered to each individual. Furthermore, it could be important to design decoys based on pathogen entry factors that are tailored to children's genetic backgrounds and prevalent strains in a given area. For instance strain N155 recognizes hosts' lacto-N-biose and N-acetyllactosamine and affects Indian newborns only.[15] Blocking these receptors with suitable decoys would likely antagonize entry of strain N155. Hence, tailored medications could be administered to genotyped children days before vaccination, therefore reducing the incidence of gastroenteritis prior to vaccination (and increasing the efficiency of immunization) while favouring entry of vaccine, rather than pathogenic strains. It would be necessary to evaluate ideal dosing/half-life of decoys as well as to consider whether vaccines are being administered during high or low Rotavirus season. Having ascertained the genetic makeup of local populations regarding Rotavirus entry factors, it would be equally important to monitor the strains that naturally occur in any given region, as is the case for annual influenza immunization campaigns. Dominant strains would continue guiding the design of new vaccines and their deployment would be adjusted to the geographic areas and patients in question.

Improving vaccine targeting to M cells with existing or novel ligands (including small molecules) using better M cell culture systems[68] and vaccine encapsulation strategies[65,67] would likely raise immunization efficiency in developing nations. In that regard, old and potentially new ligands could be validated in vitro using translational fusions or conjugations to pertinent (delivery) systems such as lactic acid bacteria, subunit vaccines, synthetic nanoparticles and VP4 (VP5*/VP8*) Rotavirus spike head protein. These findings would be extended to in vivo assays using appropriate preclinical models, with the most promising leads being evaluated in clinical trials.

Based on the outcome of 'omics' analysis regarding mucosal immunity, it may be possible to hypothesize on candidate mucosal adjuvants that could lead to increased antigen-specific cell based immunity and also IgA secretion in the gut. These could potentially be administered via intradermal route using e.g. microneedles, therefore bypassing the constraints associated with enteropathy and the requirements for a cold chain.[101]

Alternative, potentially low-cost strategies for vaccine production and storage such as transgenic plants seem to be gaining traction as exemplified by the oral delivery of rice expressing cholera toxins.[70] In principle, this approach could also be explored for Rotavirus vaccination.

Conclusions

In the future we expect significant developments in a number of fields that are likely to shape Rotavirus vaccine development. New data regarding critical Rotavirus entry factors and their distribution across populations, especially in low income areas where current vaccines underperform, will probably emerge. Further validation of technologies comprising polymer encapsulation and M cell targeting of vaccines is expected, which will likely benefit from improved cell culture methods. Systems analysis of Rotavirus vaccination, infection, mucosal pathology and immunity should help clarifying important questions regarding interventions to mitigate EE, testing and validating suitable mucosal adjuvants, key pathways involved, as well as circadian regulation of Rotavirus entry factors and response to vaccine. Progress is also expected in alternative delivery methods, formulation and thermostability of vaccines. Overall, we foresee that advances is these

fields will translate into a promising new generation of vaccines which could enter clinical trials in the coming years.

Key issues

- Rotavirus gastroenteritis is the leading cause of diarrheal death, with an estimated annual toll of 200,000 children
- Disease occurrence is modulated by access to clean water, sanitation, climate, poverty and nutrition, all of which contribute to environmental enteropathy
- Current vaccines are widely deployed, regarded as safe but show inferior efficacy in low income nations where the incidence of disease is highest
- More efficient vaccines are required to address the needs of poorer countries
- Research in areas such as mucosal immunity, adjuvants, systems vaccinology, delivery and stability will likely shape next generation Rotavirus vaccines

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