Treatment and Prevention of Human Rotavirus (HRV) in Developing Countries: The Potential of Avian Immunoglobulin Y

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A Senior Thesis submitted in partial fulfillment
of the requirements for graduation
in the Honors Program
Liberty University
Spring 2016

This Senior Honors Thesis is accepted in partial
fulfillment of the requirements for graduation from the
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Abstract

Rotavirus gastroenteritis is a leading cause of childhood mortality, killing ~1400 children younger than five daily, primarily through severe diarrheal dehydration. Eighty-five percent of this mortality occurs in developing countries where rotavirus vaccines are not widely implemented and are only partially effective. In those countries, it has proven difficult to implement the recommended supportive therapies like oral rehydration therapy (ORT) on a wide scale due to lack of both medical infrastructure and private economic investment combined with cultural bias against ORT. IgY targeting human rotavirus (anti-HRV IgY) shows potential as a passive immunotherapy that reduces rotavirus-associated morbidity and mortality, augments vaccine and ORT implementation measures, and, with international and local investment, overcomes the current barriers to rotavirus treatment and prevention.

Treatment and Prevention of Human Rotavirus (HRV) in Developing Countries:

The Potential of Avian Immunoglobulin Y

What is Rotavirus?

Under a microscope rotavirus appears playful in its spiked symmetry of 20 faces and might be mistaken for a creative mathematical creature from *The Phantom Tollbooth*, an imaginative children's novel. As innocent as it appears, this triple-layered, icosahedral particle is one of the leading causes of childhood death from gastroenteritis. Rotavirus causes the most diarrheal hospitalizations of children in the world and kills about 500,000 children every year, or about 1440 children daily. This occurs principally in developing countries where current vaccines are less prominent and seem to be less effective (see Fig. 1) (*1*, *2*).

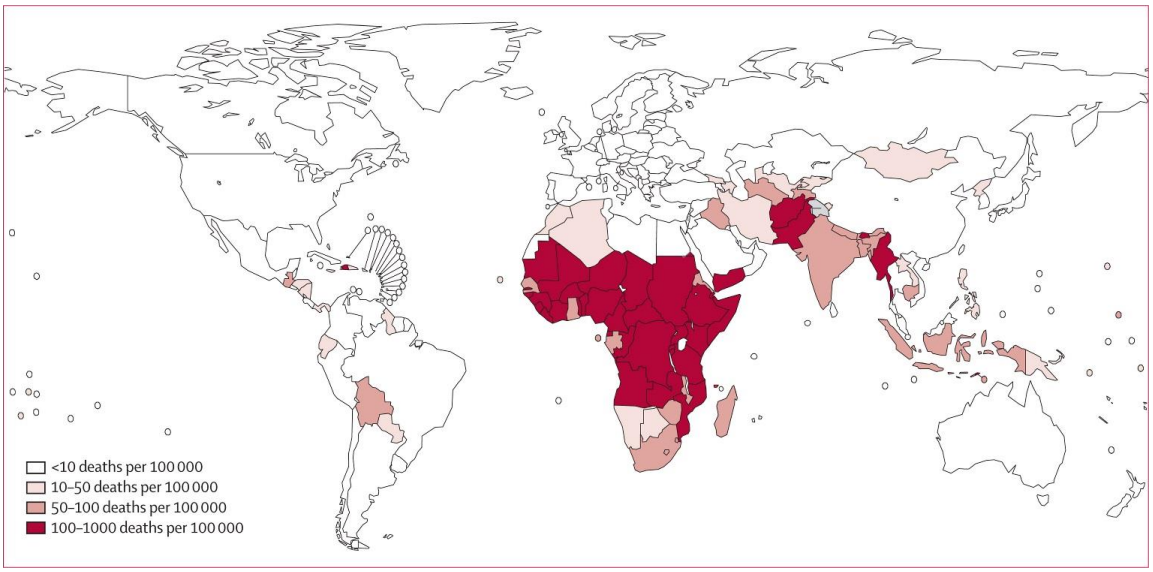

Fig. 1. Distribution of global mortality due to rotavirus in children under the age of 5 in 2008. Figure illustrating the distribution of mortality due to rotavirus in children younger than five-years-old. The darker regions represent higher mortality per 100,000 children than the lighter regions.

Reprinted from J. E. Tate *et al.*, 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. *The Lancet. Infectious diseases* 12, 136 (Feb, 2012), with permission from Elsevier.

Rotavirus's resilient structure and severely dehydrating symptoms make it a powerful childhood killer. Individual rotavirus particles are incredibly stable and remain infectious after being outside the human digestive tract for some time (weeks to months) (*3*). Rotavirus also has a very low infectious dose of less than 100 viral particles (*3*). After establishing infection, rotavirus causes severe diarrhea and vomiting, leading to significant loss of bodily fluids and resulting in dehydration. If unable to rehydrate soon after symptom onset, the children die. Low-osmolarity oral rehydration therapy with zinc supplementation is currently the recommended way to prevent death from diarrheal dehydration (*4*). In the case of rotavirus, there is no therapy currently distributed that actually targets the infectious agent. Antibiotics are not effective against viral infection and no rotavirus anti-viral is available.

Rotavirus Structure

The rotavirus virion is composed of three layers. The innermost core contains a shell composed of viral protein (VP)2, which packages the double-stranded viral RNA genome, VP1, an RNA-dependent RNA polymerase, and VP3, a guanalyltransferase and methyl transferase that forms the transcription complex with VP1 (see Fig. 2) (*5*, *6*). The middle layer of the particle is composed of VP6, while the outer layer is composed of a VP7 shell with spikes of VP4 (*6, 7*). The rotavirus replicates and assembles in structures called viroplasms that are created by the virus in the cytoplasm of the host cell, near the cell nucleus (*8*). The core and middle layers assemble in the viroplasm and then bud through the endoplasmic reticulum (ER), acquiring the outer layer in the process (*9*). It seems that rotavirus exits the apical portion of epithelial cells via an atypical vesicle

budding that bypasses the Golgi apparatus. However, release may also be lysis mediated (*9, 10*).

The rotavirus genome codes for a total of 12 proteins, the six structural proteins (VP1-VP4, and VP6-VP7) that create the virion and six non-structural proteins (NSP1-NSP6) that aid in various aspects of infection and replication inside the cell. The six non-structural proteins have unique responsibilities. NSP1 seems to play a large part in the virulence of the virus by countering many innate immune defense mechanisms. NSP2 and NSP5 are components of the viroplasm; NSP5 specifically recruits proteins to the viroplasm (*8, 11*). NSP3 assists in shutting down host RNA translation and directs machinery to promote viral RNA translation (*12*). NSP4 acts as an enterotoxin which promotes secretory diarrhea and dissemination of the virus throughout the gut via various mechanisms still under investigation (*6, 13*). NSP4 also acts as the transmembrane ER receptor to which the double layered particle binds, initiating the budding through the ER to acquire the final layer of the virion. The last non-structural protein, NSP6, is the least

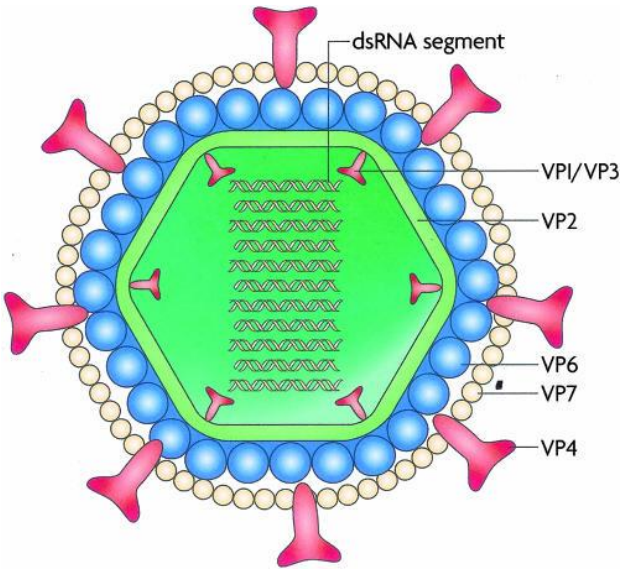

Fig. 2. Illustrated cross-section of a rotavirus. The three layers of the rotavirus encapsulate the 11 segments of double stranded RNA genetic material (dsRNA). The inner layer is composed of VP1, VP2, and VP3. VP6 makes up the middle layer, while VP4 and VP7 comprise the outer layer. As the outermost antigens, VP4 and VP7 are the principle neutralization antigens (targets for antibodies to bind and inhibit virulence). Thus, VP4 and VP7 are the main immunogenic components of the current rotavirus vaccines.

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characterized, but seems to regulate the assembly of NSP5 in the viroplasm structure and may regulate mitochondrial function during infection (*14-16*).

Currently, the VP7 shell and the VP4 spikes of the outer protein coat are known to induce a neutralizing antibody response from the immune system (*17*). These proteins are also used to identify the different genotypes and serotypes (strains of the same microorganism species with different surface antigens) of human rotavirus. VP7 is a glycoprotein and determines the G-type of the virus (e.g., G1, G2), while VP4 is a protease-activated protein and determines the P-type of the virus (*17*). Because the rotavirus genome contains 11 segments of double stranded RNA, there is a large amount of genetic reassortment when more than one strain of rotavirus simultaneously infects an individual (*18*). Thus, there are many different combinations of G and P types. However, 90% of circulating rotavirus is comprised of five strains: G4P4, G1P8, G3P8, G4P8, G9P8 (*17*).

Epidemiology of Infection

Human rotavirus is transmitted principally via a fecal oral route and seems to be only transmitted through humans. The principal reservoirs of infection are the human gastrointestinal tract and stool of infected individuals (*3*). Approximately 2 days before the start of diarrhea, the individual begins shedding significant amounts of virus in stool. That shedding continues for up to 10 days after the onset of symptoms in healthy individuals and longer (up to 30 days) in persons with compromised immune systems (*3*). In developing countries, most rotavirus infections (75%) occur prior to the age of one (*17*).

The Need for Prevention and Therapy

Since rotavirus is highly infectious and quite resilient, it is practically impossible to prevent exposure and infection solely through sanitation. The high incidence of rotavirus infection is astonishing and essentially all children are infected by rotavirus by the age of five in both developing and industrialized countries (*17*). This global, cross-cultural incidence of infection indicates that simply increasing hygienic practices in developing countries will not prevent children from becoming infected with rotavirus. Rather, a method of decreasing the severity of infection and assisting the body in fighting the disease seems to be the best way to combat this childhood killer.

The Theory Behind Vaccination

The concept of vaccination is based on the natural development of immunity where initial exposure to a pathogen allows a faster, more efficient immune response upon secondary exposure to the same pathogen. A vaccine mimics the initial infection, priming the body with a weakened form of a pathogen so that the body is prepared to defend itself against subsequent infection with the fully virulent pathogen.

Studies in Mexico (1996) and Guinea-Bissau, West Africa (2002) initially indicated that the first infection with rotavirus is the most severe followed by significantly reduced severity of subsequent infections (*19*). The first infection was found to be about 70% effective in preventing additional instances of rotavirus diarrhea. These findings support the hypothesized value of a rotavirus vaccine. Thus, scientists have worked to formulate vaccines with live, attenuated rotavirus that is capable of causing a mild, natural infection and inducing the associated immune response from the host. Then,

when the child is infected by the stronger, wild-type rotavirus, the body is prepared to respond to the infection and prevent severe gastroenteritis.

RotaShield

In 1998, a rotavirus vaccine using a rhesus (monkey) human reassortant strain of rotavirus (known as RotaShield) was licensed in the United States after demonstrating 82-91% efficacy against severe gastroenteritis caused by rotavirus (*17*). Reassortant strains arise when two different strains of a virus infect the same cell and the genetic material from both strains recombines in different ways to produce a new strain with a mixture of the genetic material from the two parental strains. This reassortment can be induced in a laboratory to create viral strains that are less infectious but contain the key human rotavirus epitopes and induce a protective immune response. RotaShield was a tetravalent vaccine and contained four reassortant strains presenting the most common G types, G1-4 (*20*). G1, G2 and G4 were human rotavirus serotypes substituted into rhesus virions, while G3 was expressed only as a rhesus rotavirus serotype (*21*). However, RotaShield was quickly withdrawn from the market after a clear association with intussusception (where one portion of the intestine folds into another portion, a sort of telescoping of the intestine) was discovered during its first year of licensure (*17*). Experts determined that the rate of association was approximately 1 occurrence per every 10,000 vaccinated children (*20*).

RV5 and RV1

Fortunately, two other vaccines, RV5 (known as RotaTeq®) and RV1 (known as Rotarix®) were licensed in the US in 2006 and 2008, respectively, after undergoing extensive clinical trials (*22*). RV5 is a pentavalent vaccine containing five bovine-human

reassortant strains of rotavirus expressing human G1-4 as well as P1A[8]; the immunogenic importance of VP4 (which determines the P type of the virus) was realized after the development of RotaShield and human VP4 was not included in the first vaccine (*20*). RV5 is distributed in 2mL vials with the viruses suspended in a buffer solution (*3*). Each vial contains approximately $2x10^6$ infectious units (IUs) of each of the five strains of rotavirus. The United States Advisory Committee on Immunization Practices (ACIP) recommendations for administration advise a series of three oral doses starting at 2 months old and repeated at 3 and 4 months with a minimum of 4 weeks between doses (*23*). They recommend that the maximum age for the first dose of RV5 is 14 weeks and 6 days, and the maximum age to complete the series is 8 months.

In 2008, the monovalent RV1 vaccine was also licensed for use in the US (*22*). A naturally attenuated (rather than laboratory attenuated or weakened) human strain of rotavirus, the P1A[8]G1 strain, contained in RV1 includes the most common human serotypes of VP7 and VP4 (*20*). RV1 is distributed as a freeze-dried powder which is reconstituted in a liquid immediately before oral administration in 1mL doses (*3*). Each dose contains 10^6 IUs of the one rotavirus strain. According to the ACIP, RV1 should be administered in a series of two doses, 4 weeks apart (*23*). The appropriate age range for administration of RV1 and RV5 is the same according to the ACIP, but the label recommendations for administration from the vaccine developers vary slightly (*3*).

116E

From 2011 to 2012, clinical trials were performed using a new monovalent human-bovine natural reassortant strain of rotavirus, named 116E, as a vaccine (*21*, *24*, *25*). This G9P[11] strain is a unique, naturally attenuated reassortant strain that was

discovered and isolated as a vaccine candidate because it induced asymptomatic rotavirus infection in neonates in India (*25*). The 116E vaccine contains the bovine P gene and 10 other human genes (*24*). It is administered in three doses at about 6, 10, and 14 weeks of age. Each dose is administered 5-10 minutes after the administration of 2.5ml of a citrate bicarbonate buffer to raise the pH of the harsh gastric environment (*24*).

Vaccine Efficacy

In various high- and middle-income locations in Latin America, Europe, and Asia, clinical trials found RV1 to be highly effective (85%-96%) against severe rotavirus gastroenteritis through the second year of life (*17*). RV5 was found to have 98% efficacy against severe rotavirus gastroenteritis and 74% efficacy against any severity of rotavirus gastroenteritis caused by serotypes G1-4 in the United States and Finland (*17*). Over the long-term, both RV5 and RV1 have proven highly effective in preventing rotavirus gastroenteritis caused by all strains of human rotavirus in the United States (since 2006 and 2008 respectively) and in other developed countries (*17, 26*).

In middle-low and low-income countries, however, clinical trials of RV1 and RV5 demonstrated much lower efficacy rates and were found to be only 51%-64% effective against severe rotavirus gastroenteritis. The reasons for this are under investigation. The middle-low and low-income countries evaluated included the African nations of South Africa, Malawi, Kenya, Ghana, and Mali, and the Asian countries of Vietnam and Bangladesh (*17*). Further, trials utilizing RV5 demonstrated substantially reduced protection in the second year of life (*19*). For maximal impact, rotavirus vaccines need to be effective through the second year of a child's life (*17*).

For 116E, the vaccine exhibited an efficacy of 56% against severe rotavirus gastroenteritis in the first year of life, while the efficacy in the second year of life was found to be about 49% (*27*). The vaccine was also not linked to a significantly increased risk of intussusception. While this is promising, the clinical trials were not performed on a large enough sample size to detect low-risk associations of vaccination to intussusception or other adverse side effects. Thus, to ensure safety, further evaluation and surveillance are warranted as the vaccine is implemented. (*28*).

In 2009, in response to the results of clinical trials of RV1 and RV5, the World Health Organization (WHO) recommended global integration of RV1 and RV5 into immunization programs (*22*). Regardless of their varying levels of efficacy, it remains clear that implementation of rotavirus vaccination on a global scale would significantly reduce the childhood death due to this disease, likely saving the lives of about 200,000 children annually.

Impediments to vaccine implementation

Even though the WHO recommended global incorporation of rotavirus vaccines into immunization programs in 2009, as of November of 2015, only five of the 15 countries with the highest mortality were using the rotavirus vaccine(s) (*29*). In order to preserve life, it is important to understand the impediments to vaccine implementation faced by developing countries. First, there is a significant cost issue (*30*). In order for manufacturers to produce the rotavirus vaccines, they performed extensive research and development. Further, due to the intussusception associated with RotaShield, RV5 and RV1 underwent extremely extensive clinical trials to prove their safety and efficacy. This

means that the newer rotavirus vaccines must be sold at a higher price to ensure adequate profit while the manufacturers control the market.

When RV5 and RV1 reached the market, they were 132 and 90 times more expensive than the most inexpensive vaccine included in the Expanded Program on Immunization (EPI) facilitated by the WHO (*30*). Fortunately, in 2011, both manufacturers offered to provide their vaccines at reduced price to the countries eligible for the Global Alliance for Vaccines and Immunizations (GAVI) funding. For those countries, manufacturers are offering RV1 at US $2.50 per dose and RV5 and US $3.50 per dose (*30*). However, even at these reduced prices, rotavirus vaccines are still one of the most expensive vaccines in the EPI. Compounding the issue are implementation costs, including the cost of training for effective vaccine administration, crucial cold storage, surveillance, etc. (*29, 30*). Thus, cost is a significant impediment to implementation in developing countries with limited financial resources and infrastructure.

The company in India manufacturing the new 116E vaccine has committed to make its doses available at a price of $1 per dose which is far more affordable than the other vaccines provided to GAVI countries (*28*). However, GAVI countries receiving international funding for vaccines only co-finance 40 cents per full vaccine series (two or three doses) of RV1 or RV5 through the program—a supplemented price that is significantly lower than the full 116E pricing (*28*). This presents two issues: first, it is not clear that many countries would be able to afford the 116E vaccine without additional funding assistance. Further, if GAVI countries choose the RV1 or RV5 vaccine while they can qualify for GAVI funding, it is unclear if they will have the ability or initiative

to continue providing rotavirus vaccination once they are responsible for paying for the vaccines without GAVI assistance (*29*).

Impediments to vaccine efficacy in developing countries

In addition to the practical/financial challenges to global vaccine implementation, recent research in developing countries indicates that there may be significant physiological impediments to vaccine efficacy in these locations. One inhibition to rotavirus vaccine efficacy could be trans-placental anti-HRV IgG. A trial performed in 2013 showed that high levels of maternal IgG crossing the placenta to the child led to diminished immune response when the children were given a live oral rotavirus vaccine (116E) (*31*). However, this trial also found that the diminished immune response could be overcome by increased vaccine dosage.

Studies in 2010, 2011, and 2013 involving breastfeeding women across multiple countries all found that women in developing countries had the highest titers of antibodies capable of neutralizing rotavirus vaccines strains (*32-34*). The 2013 study also indicated that other elements of maternal breast milk may play a role in decreasing the vaccine's ability to induce an immune response in the infant. However, another study where mothers were encouraged to withhold breastfeeding for thirty minutes prior to and after vaccine administration found no additional vaccine efficacy when breastfeeding was withheld (*35*). The withholding period was short, so the significance of these findings is not entirely clear.

There is also mixed evidence regarding the interference of live oral polio vaccines (OPV) with rotavirus vaccine efficacy. The rotavirus vaccine does not harm the immunogenicity of OPV, but studies do indicate that concurrent administration of OPV

and the oral rotavirus vaccine interferes with rotavirus vaccine immunogenicity at least for the first dose. However, this lowered immune response seems to be overcome by subsequent doses of the vaccine series. While immunogenicity seems to be about the same at the end of a full dosage of RV1 or RV5 administered with or without the live oral polio vaccine, the actual protective efficacy may not be the same in developing countries. It is unclear if vaccine immunogenicity directly correlates with protective efficacy in those countries, so experts believe this question warrants further investigation (*36*).

A study in India published in 2011 found that even natural rotavirus infection did not lessen the intensity of rotavirus-induced diarrhea until a child was infected two or three times (*37*). For the most common strain of rotavirus, G1P[8], even reinfection with the same strain (homotypic infection) was not any less severe than heterotypic infection. The protection of three natural infections against symptomatic rotavirus infection finally reached about 80% against moderate or severe diarrhea. Since vaccination is based on the observation that a host responds more efficiently to a pathogen after the first infection, this finding raises questions in the context of certain countries about the validity of the ideological basis for the efficacy of vaccination against rotavirus. The reasons for this reduced natural protection are unclear, but reduced protection could be due to a significantly higher viral titer and earlier age of infection in developing countries (*19*).

A further problem for vaccination arises when immune systems are compromised. In patients with severe combined immunodeficiency (SCID) for example, the live rotavirus vaccine can cause actual rotavirus infection (*38*).

Ultimately, the limited efficacy of presently licensed (and in process) rotavirus vaccines in developing countries means that even with 100% implementation, hundreds

of thousands of children would likely still die annually from rotavirus. Clinical trials demonstrated an efficacy of 48% for pentavalent vaccine in developing countries in Asia (Vietnam, Bangladesh) and an efficacy of 39.3% in developing countries in sub-Saharan Africa (*39, 40*). As mentioned earlier, the efficacy of the new 116E was similar, demonstrating about 50% efficacy in India where its clinical trials were performed (*24*). In addition to limited efficacy once administered, three compounding factors make it difficult to realize full vaccination benefits. First, according to the recommended regime, vaccine sequences must be completed in the first three to eight months of a child's life (depending on the vaccine used), thus a child must be brought on a regular basis, early on in life, to a place where the two or three dose vaccines are administered. Second, there will always be a subset of the population that is immune compromised and thus incapable of using the live vaccines and potentially hurt by them. Lastly, for the full public health potential of a vaccine to be realized it would need to actually prevent rotavirus infection not simply prevent the severe gastroenteritis (*22*). Avoiding severe symptoms is beneficial, but infection, viral proliferation, and viral shedding still occur, allowing the spread of rotavirus. Thus, unless infection is completely prevented, human rotavirus will continue to infect and possibly kill those who cannot be or are not protected by vaccination.

Treating Rotavirus: The Current Recommended Approach

Currently, rotavirus is treated symptomatically; there is no treatment targeting the actual infection. Current treatments target symptoms of gastroenteritis, principally dehydration which serves as the primary mechanism of mortality caused by rotavirus

infection. Oral rehydration solution combined with zinc supplementation is the most effective combination known to date able to treat/prevent dehydration due to diarrhea (*4*).

Oral Rehydration Solution

Oral rehydration solution (ORS) is composed of glucose and electrolytes that help the body absorb water through the digestive tract. In 2004, the WHO and the United Nations Children's Emergency Fund (UNICEF) issued a joint statement on the treatment of acute diarrhea, noting that a reduced-osmolarity rehydration solution (with less glucose and salt) was more effective than the original ORS in promoting hydration and reducing diarrhea and vomiting (*41*). The lower osmolarity formulation aids the absorption of water into the body by reducing the concentration of the solutes. According to WHO and UNICEF recommendations, the reduced osmolarity ORS is composed of sodium chloride (2.6 g/L), glucose, anhydrous (13.5 g/L), potassium chloride (1.5 g/L), trisodium citrate, dihydrate (2.9 g/L) (*41*).

Zinc Supplementation

Zinc supplementation has been very beneficial to the treatment and prevention of diarrhea in children in developing countries (*42-44*). In most cases, 20mg of zinc supplement per day (10mg for children under 10 months) for 10-14 days reduces the frequency and duration of diarrhea and reduces the incidence of diarrhea for about two to three months following the supplementation period (*41*). However, there are conflicting results from studies specific to rotavirus, with some finding that zinc supplementation was not efficacious in children with rotavirus infection/co-infection while other studies in China indicate that zinc supplementation is beneficial to treat rotavirus-induced diarrhea (*44, 45*). There does seem to be a role for zinc ions in rotavirus protein stability, so this

may be one reason why zinc supplementation may not be as effective in reducing rotavirus-induced diarrhea (*46*).

Intravenous Rehydration

In cases of severe diarrheal dehydration (SDD), the WHO manual on the treatment of diarrhea recommends slightly different guidelines for addressing dehydration. Instead of oral rehydration therapy, WHO recommends rapid intravenous rehydration administered in a hospital (*47*). The recommended treatment plan suggests administering 30ml/kg in the first hour (for infants under 12 months) or 30 minutes (for older children) followed by administration of 70ml/kg in 5 hours (or 2.5 hours for children older than 12 months). However, in SDD a variety of other complicating factors affect the efficacy of treatment (*48*). In particular, many children with SDD suffer from hypokalemia (low levels of potassium in the blood stream) and/or hypernatremia (high levels of sodium) (*48*). Both of these situations are common and neither is well addressed by the typical intravenous solution such as Ringer's lactate solution, which actually contains high sodium and low potassium concentrations (*47, 48*). WHO recommends administering some ORS even while the child is severely dehydrated to try to balance these electrolytes (*47*). Studies also indicate that ORS is more effective than intravenous solutions alone for stabilizing electrolyte imbalances (*49*). Thus, it is clear that the sooner the child can receive ORS the better. Unfortunately, in under-resourced settings, IV therapy may not even be available. With this in mind, the importance of stabilizing the child so that he or she is able to retain ORS without nasogastric administration is clear and is therefore a key goal of any rotavirus treatment regime applicable to the highest burden areas.

Impediments to Implementation and Efficacy

A 15-year UN initiative started in 2000, the Millennium Development Goals, had as its fourth goal (MDG4) the reduction of under-five mortality by two-thirds from what it was in 1990 (*50*). While almost able to achieve MDG4 with an approximately 50% reduction, in 2015 about 5.9 million children under the age of 5 still died. Diarrhea alone accounted for 9% of these deaths (*29, 50*). Of specific interest is the fact that the proportion of severe diarrhea (and likely death) due to rotavirus relative to the total of severe, hospitalizing diarrhea has at least doubled over the past 15-20 years (*1*). In 2013, recognizing that the 2015 MDG4 was far from being reached for many of the poorest countries, UNICEF and WHO set forth a Global Action Plan for the Prevention and Control of Pneumonia and Diarrhoea (GAPPD). The GAPPD set forth a goal of 90% coverage with ORS and zinc treatment for diarrhea of 15 target countries with the highest mortality due to pneumonia and diarrhea, the two leading childhood killers, by 2025. Additionally, the GAPPD also established the Sustainable Development Goal 3.2 of ending preventable deaths of infants and children by the year 2030 (*51*).

As anticipated, by the end of 2015, ORS and zinc coverage was still depressingly low. Only two of the countries had achieved an ORS coverage of over 50% for children with diarrhea and none of the countries had coverage over 11% with zinc supplements (*29*). While implementation of coverage of both pneumonia and diarrhea interventions was much lower than hoped, countries consistently had more difficulty implementing diarrhea interventions. Over all, in the countries experiencing the highest morbidity and mortality due to rotavirus, only about 34% of children with diarrhea receive any type of ORS (original or low-osmolarity) and less than 5% receive any sort of zinc

supplementation (*4*). Further, international attention has shifted to other diseases like HIV and malaria which kill fewer children under the age of 5 than "simple" diarrhea and pneumonia but have more of a dramatic treatment appeal.

Practical impediments. Bangladesh is one country that has successfully scaled up its ORS and zinc coverage of diarrhea. Unger et al. identified a strong private-sector pharmaceutical industry and in-country production as being key to its success in developing and implementing ORS and zinc treatments (*4*). In the 2015 assessment of progress towards GAPPD goals, the International Vaccine Access Center identified three success factors in countries that had sustained greater than 50% coverage of ORS for three years. These factors included (i) both public and private sector engagement and stakeholders, (ii) integrated efforts to increase demand in both the public and private sector, and (iii) other support factors including political stability and designated funding (*29*). It seems difficult for countries lacking these factors to successfully implement treatment.

Overall, the most recent assessments have concluded that each country faces unique challenges to diarrheal therapy implementation (*4*, *29*, *50*). Thus, each country needs to develop an individualized action plan, identifying relevant economic and cultural barriers to therapy implementation which will likely need to be addressed by the combined efforts of the public and private sector.

Cultural impediments. While each nation has its unique barriers to ORS and zinc implementation, one overarching difficulty in ORS implementation is the perception that it is not a "medicine" (*4*). ORS does not quickly reduce diarrhea and allows rotavirus to run its course but prevents death from dehydration. Thus, many caregivers demand

antibiotics or antidiarrheal medications which harm the patients more than help them. Further, the lack of access to hospitals in rural areas is a problem. When the diarrhea and vomiting become too severe for normal ORS administration, these communities are unable to access a hospital with the resources for nasogastric or IV administration of rehydration fluids necessary to save lives.

The Need for Another Therapy

In light of the current difficulties facing vaccine success and ORS implementation, "[t]he need for an approach to complement but not compete with prevention by immunization and treatment with ORS, therefore, is certain," Dr. George Fuchs, professor of pediatrics practicing in Pediatric Gastroenterology and Nutrition at the University of Kentucky, stated in an invited commentary on an adjuvant rotavirus therapy (*52*).

In 2013, when the WHO published its action plan to address pneumonia and diarrhea, a recurring theme emerged throughout its evaluation of previous successes and failures (*51*). Moving forward, the GAPPD emphasized the necessity of involvement of local communities and industry in the treatment/prevention of diarrhea alongside the support of the larger international organizations through collaborative efforts. To this end, the GAPPD states:

> The GAPPD is committed to the promotion of research, including community-based action research and sociocultural research on knowledge, attitudes, perceptions, cultural practices and health seeking behaviours.…, there will be a need for research on delivery strategies, on overcoming barriers to interventions and on better ways for implementation.… Research efforts to control pneumonia

> and diarrhoea in children must include building research capacity in the countries most affected. (pg. 34 (*51*))

Keeping in mind the need for an adjunct therapy, sensitivity to cultural attitudes and practices, and investigation augmenting local research needs and abilities, avian immunoglobulin Y (IgY) seems quite promising as a passive oral immunotherapy for rotavirus in developing countries.

Research on using IgY to treat intestinal diseases has been performed across the world for approximately thirty years. In particular, anti-HRV IgY has been a subject of research in Myanmar, India, China, Argentina, Japan and Bangladesh within the past 15 years (*53-56*). As knowledge of avian biology, immunization adjuvant systems, and IgY administration systems increased over the past decade, IgY technology has emerged as an affordable, sustainable, and effective means of producing specific antibodies. In particular, since much research on efficient production and extraction of antibodies from chickens is available to the public, IgY technology can be developed in any country by any business that wishes to do so. Thus, it is worth understanding the potential of IgY to address current gaps in the treatment/prevention of diarrheal death as a unique therapy that lends itself to both international investment and collaboration with local research facilities.

What is IgY?

Immunoglobulin Y is an antibody found in chicken egg yolks. By vaccinating chickens with a microorganism or other antigenic substance, IgY that specifically binds that microorganism is produced by the chicken and then transferred into the egg yolk. IgY is simpler and cheaper to produce than many other mammalian antibodies (which

require bleeding and careful purification from other reactive serum components) and can be used for various diagnostic assays and laboratory procedures. Since chicken eggs are a natural part of the human diet, IgY works well as a component of passive oral immunotherapy for various enteric diseases such as rotavirus. Since production and extraction is relatively easy and inexpensive and IgY is easy to store while retaining antigen affinity, IgY shows promise for development and implementation in developing countries where extensive lab equipment, special storage systems and refrigeration are limited.

Avian Immunity

As a part of their immune system, adult chickens have three immunoglobulin (antibody) classes: IgA, IgM, and IgY (*57*). In mammals, a developing embryo is protected via placental transfer of IgG and, once the young is born, further immune protection is conveyed through mucosal antibodies secreted in the breast milk (*57*). However, for birds, all immune protection must be conveyed to the chick in the egg. IgM and IgA are transferred to the chick in the egg white, while IgY is selectively transferred to the egg yolk from the blood serum in a receptor mediated process (see Fig. 3) (*57, 58*). As the only antibody class found in the egg yolk, IgY is easy

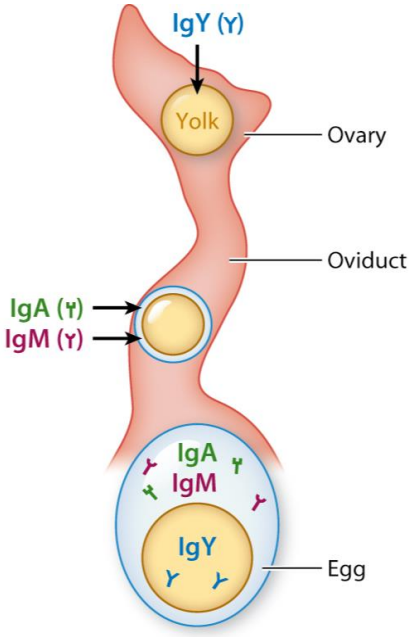

Fig. 3. Antibodies present in the avian egg. Antibodies are transferred from the hen blood serum into the developing egg. IgY is selectively transported to the egg yolk while IgA and IgM remain in the egg white.

Figure reproduced from J. Kovacs-Nolan, Y. Mine, Egg yolk antibodies for passive immunity. *Annual review of food science and technology* 3, 164 (2012).

isolated from the other immunoglobulins. On average, a 50-gram egg can contain between 100-150mg of IgY (*57*).

Antigen Specific IgY

While different breeds of laying hens may have different typical amounts of IgY, once the chickens are immunized, they all seem to contain similar amounts of IgY in the egg yolks (*59*). In order to produce IgY that binds specific antigens, the chicken must be immunized with that antigen. This is typically done intramuscularly with an initial dose of antigen, followed by a sequence of booster shots. Depending on the type of adjuvant (a substance encouraging an immune response) used, varying numbers of booster shots are needed to maintain high-levels of antibody production (*60*). Once the antibodies are produced in the chicken serum, they are transferred to the eggs rapidly with substantial quantities of antigen specific IgY present in the yolk about a month after initial inoculation (*53*). With appropriate booster immunizations, high titers of IgY can be maintained for over a year. Interestingly, older laying hens who have completed two laying cycles (considered to be "spent" for commercial production of eggs for consumption) still produce eggs with high titers of antigen specific IgY and therefore may be a particularly cost-effective source of IgY (*57, 61*).

In the case of rotavirus, research demonstrates that immunization with whole rotavirus particles initiates a polyclonal antibody response which provides more effective neutralization than immunization with one viral particle (e.g., VP6) (*56*). Successful clinical trials in 2012 utilized a "Rotamix IgY" which was produced by combining two clinical strains of rotavirus in vaccination, stimulating production of IgY against various epitopes of both strains. The Rotamix IgY was able to cross react with all major clinical

strains of rotavirus in neutralization assays demonstrating its ability to provide passive immunity against human rotavirus (*54*). Unlike vaccination (active immunity), passive immunotherapy does not require a strong host immune system. The antibodies from the donating organism (in this case a chicken) neutralize the infectious agent in the recipient host (see Fig. 4).

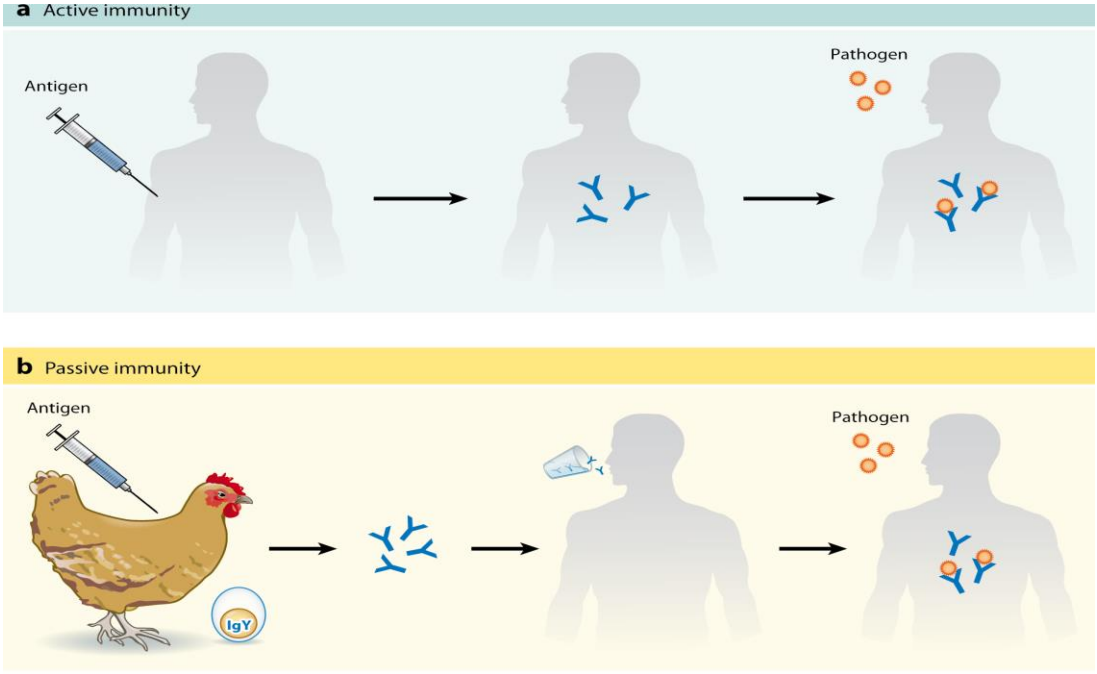

Fig. 4. The difference between active and passive immunity.
(a) Active immunity, induced by rotavirus vaccination of humans, requires a host immune response to fight the pathogen. (b) Passive immunity, provided by avian IgY, does not require a host immune response to neutralize the pathogen. This is particularly beneficial when the host immune system is weakened or not well-developed.

Figure reproduced from J. Kovacs-Nolan, Y. Mine, Egg yolk antibodies for passive immunity. *Annual review of food science and technology* 3, 165 (2012).

In the past decade, various adjuvant systems have been tested to determine the most efficient way of stimulating a thorough avian immune response to the vaccination. Typically, incomplete Freund's adjuvant (IFA) is used with vaccinations in order to promote an immune response but not damage the tissue of the animal. A 2007 study showed that C-phosphate-guanosine-oligodeoxynucleotide (CpG-ODN) supplementation

of IFA was cost-effective and increased the yield of antigen-specific IgY (compared to IFA alone) by an average of 430% in each egg with a maximum increase of 942% (*60*). CpG-ODN mimics a common component of bacterial membranes and induces a strong immune response. In milligrams, this increase in IgY yield equates to 96mg of antigen specific IgY per egg (*60*). With a conservative estimate of 5 eggs per week, one laying hen could produce about 25,000mg of antigen specific IgY in one year. Since most laying hens produce an average of 325 eggs per year, one hen could produce closer to 30,000mg of specific IgY annually (*60, 62*). In the 2012 clinical trial, pediatric patients were treated successfully with about 30mg of HRV-specific IgY daily as an adjunct to standard supportive therapy (dosage clarified through personal correspondence with Dr. Shofiqur Rahman, February 8, 2016) (*54*). Even with a higher estimate of 100mg of antigen specific IgY administered daily, one laying hen could hypothetically produce 250 daily quantities of pediatric rotavirus treatment annually.

Isolation of IgY

After separation of the yolk from the egg white, IgY must be extracted from the rest of the yolk lipid and protein components. There are a variety of safe, cost-effective methods that can be used to extract IgY from the rest of the yolk contents using solvents such as polyethylene glycol (PEG) and salts such as ammonium sulfate to precipitate IgY (see Appendix A) (*62-64*). In a particularly simple method, high-purity, high-yield, high-activity IgY (98.7% pure, at 80.9% yield, with 96.3% activity) was separated from the yolk using a sequential method without the use of toxic compounds or organic solvents (see Fig. 5) (*64*). In this same protocol, another protein in the egg yolk, phosvitin, which demonstrates antibacterial properties, is extracted from the pellet parallel to IgY

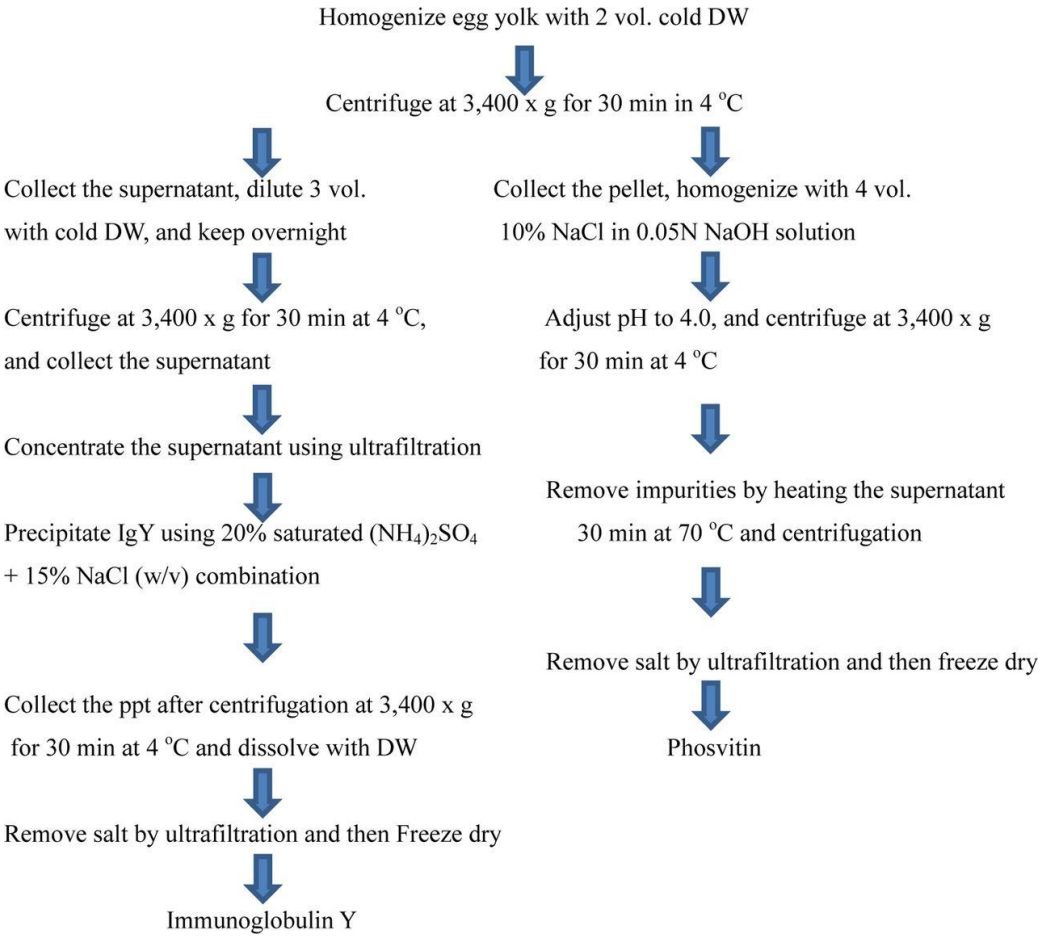

Fig. 5. Schematic diagram of the sequential separation of IgY and phosvitin from egg yolk. An outline of the simple, sequential process of isolating both IgY and phosvitin from the chicken egg yolk. This method result in an IgY product that is 98.7% pure, at 80.9% yield, with 96.3% activity.

H. Y. Lee, E. D. N. S. Abeyrathne, I. Choi, J. W. Suh, D. U. Ahn, Sequential separation of immunoglobulin Y and phosvitin from chicken egg yolk without using organic solvents. *Poultry Science* **93**, 2675 (October 1, 2014). Reproduced by permission of the Oxford University Press.

extraction from the supernatant (the liquid portion of the yolk sample following centrifugation) by a similar sequential method.

Storage of IgY

There are a variety of ways that eggs can be stored and retain active IgY in their yolks. Eggs can be refrigerated at 4°C for at least 6 months, stored at typical commercial refrigeration temperatures (10-12°C) for at least two weeks (this was the only period tested), or stored at room temperature for at least one month (*60, 65*). Once isolated, IgY

can be stored in a variety of ways; for example, it can be suspended in phosphate-buffered saline (PBS) and frozen while retaining activity for at least a year (*62*). If IgY is freeze-dried with low levels of sugars, studies indicated that the powder is stable at room temperature for a year or more retaining almost all of its activity (*66*). If freeze dried without a sugar, IgY is stable for at least three months and retains most of its activity. IgY retains its activity at temperatures up to 70°C, high pressures (up to 4,000 kg per cm^2) and can be stabilized further against temperature and pressure using various sugars as mentioned above (*67*).

Unique Characteristics of IgY

Like all antibodies, IgY has an antigen binding region (F_{ab}) and a constant (F_c) region which binds F_c receptors on cells activating the appropriate immune response (inflammation, chemotaxis, fever, etc.). While IgY is similar in function to the mammalian IgG, the F_c region of the antibody is unique thus allowing it to bind antigen without inducing an additional response via the complement system, or bacterial or mammalian F_c receptors (see Fig. 6)

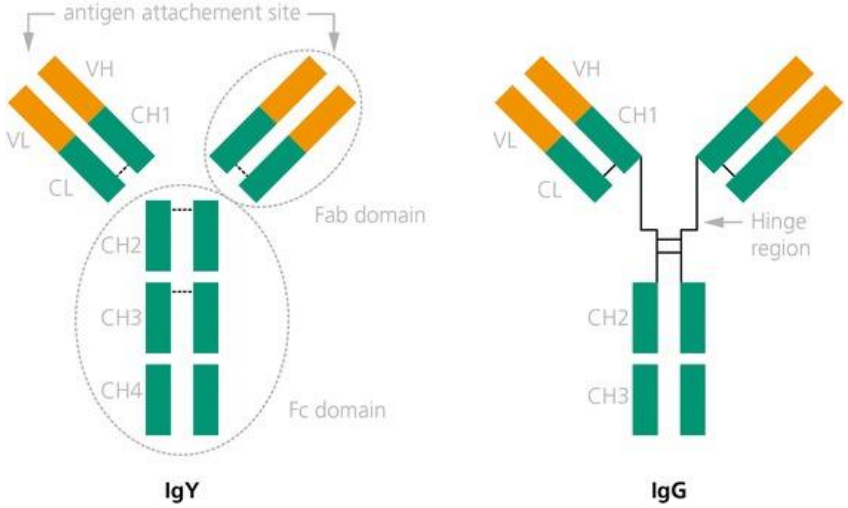

Fig. 6. Comparison of IgY and IgG.
Both avian IgY and human IgG contain the usual F_{ab} (antigen binding) and F_c (constant) regions, the differences between the two are significant enough that avian IgY does not activate the mammalian immune response to IgG.

Figure reproduced from: S. Muller, A. Schubert, J. Zajac, T. Dyck, C. Oelkrug, IgY antibodies in human nutrition for disease prevention. *Nutrition journal* 14, 109 (2015).

(*58, 67-69*). It also does not usually permeate the gut barrier or enter the blood stream. However, in a neonatal piglet model using a crude extract of IgY, some anti-IgY IgG was found in the piglet serum (*56*). Assessing the presence of anti-IgY IgE (IgE is the antibody involved in allergic reactions) is an important next step in research to ensure that an allergic reaction will not be promoted by a particular extract of IgY (*56*). However, because of the unique nature of IgY, it can be used to neutralize various microbes that infect the gastrointestinal tract of mammals without activating a severe immune response by the host immune system. IgY also has 3-5 times higher affinity for antigens than mammalian IgG, reacts more rapidly with antigens when compared to IgG in competition assays, and does not react with rheumatoid factors—antibodies made by an organism against the F_c portion of its own antibodies and associated with autoimmune diseases (*67*). These characteristics make IgY an ideal candidate for various immunoassays and diagnostic tests.

Safety

As a normal component of the human diet, eggs are typically very safe to consume and IgY has been assigned "Generally Recognized As Safe" status by the FDA and the USDA (*67*). In various studies, even non-antigen specific IgY has been shown to have antimicrobial and immune enhancing effects (*56*). Although egg allergies are typically stimulated by proteins present in the egg white (e.g., albumins), they are a minor concern (*67*). However, some individuals are allergic to certain yolk components, thus, in areas where those allergies are prevalent, it is likely that more purified IgY would need to be used and the potential risks weighed against the potential benefits (*56*). Two other factors also indicate that egg allergies are less of a concern for anti-HRV IgY therapy:

first, that systemic tolerance to various egg proteins can be induced by oral administration of that protein, and second, that neonatal immune systems are often protected by breast-feeding from developing allergies to oral antigens (*56, 67, 70, 71*). In one study, patients used IgY for up to 12 years with no adverse effects (*72*). Another safety concern is the development of resistant microorganisms. IgY, in comparison to antibiotic treatment, is far less likely to encourage development of resistant organisms since the antibody is usually produced against multiple epitopes of the whole organism rather than one component produced by one gene (*67*).

Treatment of Human Disease

IgY shows great potential for the treatment and prevention of various diseases (particularly in the GI tract) and even neutralization of toxins from events such as snake bites and bacterial infections (*57*). IgY has been tested as a prophylaxis for dental caries in children, periodontitis, and oral candidiasis affecting immune compromised patients, showing potential for treating all of those oral infections (*67, 73, 74*). In the stomach, IgY has been used for various aspects of treatment and prevention of *Helicobacter pylori* colonization—significant because *H. pylori* colonization leads to gastric ulcers and even cancer and is becoming resistant to common antibiotics (*58, 67, 75-77*). IgY is also a candidate for treatment of Celiac disease and inflammatory bowel disease (*57, 58*). In addition to the GI tract diseases, acne-causing bacteria is a target for IgY therapy since IgY is easily incorporated into face-washes, creams, or cosmetic products (*78*).

One of the most informative studies regarding the viability and safety of IgY to treat human disease is a long-term study of patients with cystic fibrosis (CF). Chronic *Pseudomonas aeruginosa* (PA) infections cause rapid decline in CF patients. For the

study of IgY treatment, participants used a daily mouthwash containing 50mg of anti-PA IgY in a 70mL quantity of mouthwash that each person gargled and swallowed every evening after brushing his or her teeth (*72, 79, 80*). Some patients were a part of the program for up to 12 years (as of 2008) and researchers found that IgY was just as effective as most antibiotic therapies to manage PA colonization. Further, no organisms developed resistance to IgY treatment, while it often only takes a single gene mutation for PA to exhibit resistance to many of the antibiotics used to treat CF patients. Additional damage occurs when broad-spectrum antibiotics are used, wiping out the normal flora of the patient and allowing other opportunistic infections to arise. At the end of the long-term study, patients in the IgY test group had statistically significant lower number of episodes of PA colonization combined with a lower probability of an early recurrence of chronic infection. Since the sample size was small, certain other differences between the IgY and control groups did not reach statistical significance, however the IgY group demonstrated healthier BMI, received fewer doses of antibiotics, and were not hospitalized for any lung infections during the treatment period (it was not clear if any of the control patients were hospitalized either). No adverse effects of the long-term IgY treatment were found over the entire study period—up to 12 years for some patients.

In addition to products for livestock and aquaculture industries, there are now IgY products available on the market for human consumption (*57*). These include anti-*H. pylori* urease IgY in yogurt and tablet form, a supplement containing IgY against 26 common human enteric pathogens, and an IgY mix demonstrated to enhance athlete recovery time and performance.

IgY in the Digestive Tract

Alone, IgY is active at a pH range of 3.5-11 and other substances such as sorbitol can stabilize IgY at lower pHs (*67*). In terms of its stability in the gastrointestinal tract, IgY is naturally resistant to digestion by trypsin and chymotrypsin but is more sensitive to pepsin when the enzyme is present at certain ratios and/or certain pH. However, at a pH of 5 or higher, IgY is also resistant to pepsin (*67*).

There are a few methods that can be used to protect IgY from the harsh conditions of the stomach. These include administration of IgY in a sodium bicarbonate buffer, freeze-drying with gum-arabic, encapsulation of IgY in liposomes (e.g., egg lecithin/cholesterol liposomes) and encapsulation in chitosan-alginate (*67*). Chitosan-alginate encapsulation of IgY has been demonstrated to protect the IgY from pepsin and acid in the stomach, while releasing it in the intestine to address enteric organisms (*81-83*). This method of encapsulation allowed faster clearance of a bacterial infection with IgY in a pig model. Un-encapsulated IgY was more effective than typical antibiotic treatment, but compared to the encapsulated IgY the beneficial effects were delayed by three days. In a gnotobiotic piglet model of HRV infection, IgY was administered prophylactically in solution with bovine milk, and the therapy was still effective without further preparation/processing of IgY (*56*).

Rotavirus Treatment Models

Various animal models of rotavirus infection along with clinical pediatric trials have shown that IgY is able to prevent and treat rotavirus gastroenteritis in a dose-dependent manner. In 1998, Sarker and colleagues demonstrated that passive immunization with bovine serum antibodies could successfully treat rotavirus infection (*84*). As a follow-up, Sarker et al. performed a clinical trial in 2001 using an anti-HRV

IgY solution (yolk powder dissolved in water) to treat pediatric patients with the safer, more easily extracted avian immunoglobulin (*55*). While the IgY improved viral clearance, it did not reduce the duration or severity of the diarrhea. These investigators hypothesized that this was due to low dosage of IgY. In 2007, utilizing a mouse trial of rotavirus infection, the same researchers demonstrated that the efficacy of IgY was indeed dose dependent (*85*). Further, at higher doses, IgY treatment did reduce the duration of diarrhea in the mouse model.

In 2011, Buragohain and colleagues performed extensive *in vitro* and *in vivo* studies of IgY prophylaxis and therapy utilizing IgY from hens vaccinated against the five most prevalent serotypes of HRV (1,2,3,4, & 9) (*53*). For the *in vitro* neutralization studies, it was found that each anti-HRV IgY neutralized its own strain well. Anti-HRV-3IgY was also able to neutralize all of the serotypes, while 1,2,4, & 9 were less effective on the other strains but still were able to neutralize more than one serotype. Since neutralization is a good indicator of the effectivity of an antiviral treatment *in vivo*, this indicated that IgY has promise as a therapeutic agent. As expected, the IgY from the unimmunized hens was not able to neutralize any of the rotavirus serotypes.

The *in vivo* experimentation performed by Buragohain and colleagues used a suckling mouse model of HRV infection. In this model, the post-inoculation IgY (therapeutic IgY) was effective in reducing diarrhea, viral load, and histopathological signs of infection in a dose dependent manner—the higher doses of IgY being more beneficial than the lower doses as shown by PCR and histological analysis. The mice that were fed IgY pre-inoculation with rotavirus (prophylactic IgY) were able to avoid many signs of infection at the times when infection is usually most physically visible. Viral

RNA in the intestinal lumen was undetectable and the histopathology of these mice was normal. These results indicate that avian IgY has promise as both a prophylaxis and treatment for human rotavirus infection in young children.

Around the same time, researchers in Argentina used a gnotobiotic (known-microbiota) neonatal piglet model to mimic HRV infection and treatment in neonates (*56*). Chickens were immunized with two different antigenic substances: one group was immunized with VP6, and one group was immunized with the whole particles of a prevalent strain of human rotavirus, Wa G1P[8]. The so-called Wa HRV IgY was the most effective in neutralization assays as well as in the gnotobiotic piglet experiments. A crude extract of IgY was prepared via ammonium sulfate precipitation (see Appendix A) and mixed with bovine milk for administration to the piglets. At a high enough titer of IgY, the supplemented milk fully protected the piglets from HRV diarrhea and significantly lowered the amount of viral shedding in feces. At a lower titer, the IgY reduced the impact of HRV infection and delayed viral shedding but was unable to completely prevent it. Further, the effectiveness of the treatment was correlated with the presence of active anti-HRV IgY antibodies in the feces. This result indicates that at least some semi-purified IgY extract is able to survive the GI tract intact, and perhaps indicates that protection of IgY in the harsh gastric environment via encapsulation or other treatment may allow higher efficacy when smaller quantities of antigen specific IgY are administered. The anti-VP6 IgY did not perform well in neutralization assays in the laboratory, and it failed to prevent HRV gastroenteritis. However, anti-VP6-IgY did demonstrate therapeutic benefits by lessening the severity of HRV gastroenteritis. This

may be partially due to other immune enhancing components of egg yolks which accompanied the crude IgY extract used in this experiment (*56*).

In 2012, Rahman and colleagues published the results of another clinical trial in Myanmar where anti-HRV IgY was implemented as an adjunct therapy to standard supportive treatment of pediatric patients (*54*). They administered the IgY by mixing the freeze-dried yolk powder with maltitol and banana flavoring and administering four 500mg doses of the mixture to each child in the IgY group daily. The four doses of the mixture contained a sum of about 30mg of antigen specific IgY (dosage clarified through personal correspondence, February 8, 2016). Rahman et al. found that the IgY treatment significantly reduced the duration of IV fluid administration, the amount of oral rehydration fluid intake, the duration of diarrhea and the average stool frequency. They also found that IgY treatment reduced the frequency of viral shedding (meaning that fewer rotavirus particles were spread into the environment). Patients who received the anti-HRV IgY treatment stopped needing IV treatment 3 days earlier than the control group, recovered 2 days faster from diarrhea and stopped shedding rotavirus 1 day earlier (*54*, *67*). Overall, the variety of animal models of HRV infection that have been successfully treated with various antigen-specific IgY preparations combined with promising results from most recent clinical trials in Myanmar indicate that IgY is a viable treatment for human rotavirus in a developing country setting.

IgY Addresses Current Gaps in Rotavirus Treatment

The low efficacy of current and emerging vaccines in developing countries, the inability to vaccinate immune compromised individuals (including the elderly) or children over 1 year of age who are out of the vaccine administration age range, the

difficulties of cultural barriers to ORS implementation, and difficulties of IV administration/availability all demonstrate the need for a better rotavirus treatment. It must be one that is effective, inexpensive, widely applicable and culturally acceptable. Further, it should complement local research ability and encourage economic growth of developing nations to provide local investment incentive.

Passive oral immunotherapy with IgY: (i) addresses cultural stigma by reducing diarrheal duration and severity which promotes its administration as a medicine and reduces the use of harmful antibiotics, (ii) reduces the duration of IV administration (allowing the more effective ORS to be used sooner), (iii) reduces the quantity of ORS needed for rehydration (facilitating caregiver follow-through with ORS for whole time period needed to rehydrate the child), (iv) is able to be used by all patients (immune compromised, malnourished, old or young, and those already infected with rotavirus) because it does not require the host immune system to prevent or clear the infection, (v) does not require a cold chain supply system and is easy to distribute, store and administer once manufactured, (vi) reduces viral shedding and thus the spread of infection, and has the potential (unlike vaccines) to prevent infection, thereby promoting an even greater public health benefit (*22*).

Perhaps more importantly, IgY technology development offers an opportunity for the international community to partner with research that has already been performed in these developing countries, augmenting local research capabilities and interests. IgY could be mass produced in larger cities or developed on a small scale for local use in rural hospitals which have limited resources (e.g., less IV solutions and equipment for administration, reduced cold-storage capabilities). IgY technology would be able to

evolve with each country's needs as it developed both economically and socially. IgY technologies could be shifted to address other infections (e.g., *H. pylori*, acne, PA) without promoting antibiotic resistance, or it could be channeled to production of antibodies for diagnostic assays and other research applications. Nations are much more likely to invest their limited resources into developing an industry which they see as being able to benefit their nation economically in the long run as well as in the present. Therefore, anti-HRV IgY presents a unique solution that is medically, practically and economically promising and warrants serious consideration for investment by the international community as it seeks to address the global mortality of children under the age of 5. If action is taken soon, perhaps 20-sided creatures will be limited to children's novels, bringing children's imaginations to life instead of silencing them forever.

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Appendix A

Three Methods of IgY Precipitation

Sequential Separation of Immunoglobulin Y

Resulting extract of IgY exhibited a yield of 80.9%, was 98.7% pure, and retained 96.3% activity.

1. Egg yolk was separated from egg white, diluted with 2 volumes of cold (4°C) distilled water (DW) and homogenized for 1 min using a hand blender (Kitchen Aid) at high speed (set at 9).
2. After centrifugation at 3,400 × g for 30 min at 4°C, the supernatant was used to separate IgY and the precipitant for phosvitin separation.
3. For IgY separation, the collected supernatant was diluted again with 3 volumes of cold (4°C) DW, kept in a cold room overnight to precipitate phospholipids and lipoproteins, and then centrifuged at 3,400 × g for 30 min at 4°C.
4. The resulting supernatant was concentrated using ultrafiltration (membrane filter cut-off size: 50 kD, GE Healthcare Bio-Sciences Corp., Piscataway, NJ).
5. Immunoglobulin Y in the concentrated solution was precipitated using 20% saturated ammonium sulfate (final concentration) and 15% NaCl (wt/ vol, final concentration) combination.
6. The precipitant was collected after centrifugation at 3,400 × g for 30 min at 4°C, dissolved with 9 volumes of DW, and then precipitated again with the same (NH4)2SO4 + NaCl combination to improve purity.

7. The precipitant was dissolved with 9 volumes of DW, desalted using ultrafiltration, and then freeze-dried (FreeZone Freeze Dryer, Labconco Corp., Kansas City, MO).

Source: (*64*)

Rough Extraction of IgY Using Ammonium Sulfate Precipitation

This precipitation method results in a rough extract of IgY, not a highly purified extract.

1. Dilute egg yolks in distilled water (ratio 1:3).
2. Centrifuge at 8,000g for 12 min at 4C to remove lipid.
3. Incubate supernatant with 0.24 g/ml ammonium sulphate for 10 min at RT.
4. Centrifuge at 10,000g for 12 min at 4C.
5. Resuspend the pellet in 2M ammonium sulphate solution at the original egg yolk volume and incubate at RT for 10 min.
6. Centrifuge the solution at 10,000g for 12 min at 4C.
7. Resuspend pellet in PBS (pH 7.4) at a 1:10 ratio of the original volume of the egg yolk (not the diluted solution).
8. Dialyze against PBS, sterilize by filtration (0.22um pore size membrane filter) and store at -20C until used.

Source: (*56*)

Extraction of IgY Using Polyethylene Glycol Precipitation

1. The eggshell is carefully cracked and the yolk is transferred to a "yolk spoon" in order to remove as much egg white as possible.
2. The yolk is transferred to a filter paper and rolled to remove remaining egg white, then the yolk skin is cut with a lancet or a similar instrument (pipette tip). The yolk is poured into a 50 ml tube and the egg volume is registered (V1).
3. Twice the egg yolk volume of PBS is mixed with the yolk (ΣV1+V2), thereafter 3.5 % PEG 6000 (in gram, pulverized) of the total volume is added and vortexed, followed by 10 min rolling on a rolling mixer. That step of the extraction procedure separates the suspension in two phases. One phase consists of "yolk solids and fatty substances" and a watery phase containing IgY and other proteins.
4. The tubes are centrifuged at 4°C for 20 min (10,000 rpm according to 13,000 x g, Heraeus Multifuge 3SR+, fixed angle rotor). The supernatant (V3) is poured through a folded filter and transferred to a new tube.
5. 8.5 % PEG 6000 in gram (calculated according to the new volume) are added to the tube, vortexed and rolled on a rolling mixer as in step 3.
6. Repeat step 4 with the difference that the supernatant is discarded.
7. The pellet is carefully dissolved in 1 ml PBS by means of a glass stick and the vortexer. PBS is added to a final volume of 10 ml (V4). The solution is mixed with 12 % PEG 6000 (w/v, 1.2 gram) and treated as in step 3 (vortex, rolling mixer).
9. Repeat step 6 and dissolve the pellet carefully in 800 µl PBS (glass stick and vortex). Wait for the air bubbles to disappear and then transfer (pipette) the

extract to a dialysis capsule. Rinse the tube with 400 µl PBS and add the volume to the dialysis device (V5).

10. The extract is dialyzed over night in 0.1 % saline (1,600 ml) and gently stirred by means of a magnetic stirrer. The next morning, the saline is replaced by PBS and dialyzed for another three hours.
11. Thereafter the IgY-extract is pulled from the dialysis capsule by a pipette and transferred to 2ml tubes. The final volume is around 2 ml (V6).
12. The protein content (mg/mL) of the samples is measured photometrically at 280 nm (1:50 diluted with PBS) and calculated according to the Lambert-Beer law with an extinction coefficient of 1.33 for IgY (purity and recovery are around 80%).
13. It is advisable to store the samples in aliquots at -20°C (do not freeze the samples at -70°C).

Source: (*62*)

Appendix B

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Nutr J. 2015; 14: 109. PMCID: PMC4617726
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IgY antibodies in human nutrition for disease prevention

Sandra Müller, Andreas Schubert, Julia Zajac, Terry Dyck, and Christopher Oelkrug

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Acknowledgements

Many people have invested in this project, directly or indirectly, and I am grateful for all of them. However, I would like to specifically thank:

- Dr. Kimberly Mitchell, my thesis chair, for giving me the freedom and encouragement to pursue my interests in this thesis; her academic guidance and friendship have significantly impacted my undergraduate years.

- Dr. Joseph Brewer, Associate Dean for Research at the Liberty University College of Osteopathic Medicine, for his willingness to sit on my thesis committee and invest in my academic growth; I know he had far more important things vying for his time.

- Professor Travis Holt, my third reader, for his investment in developing my written communication over these past few years. Professor Holt's classroom dynamics inspire me to communicate things of value in a manner that transcends social and cultural biases.

- Dr. Randall Hubbard, for inspiring this research in the first place and for always teaching us to always LTL first.

- Jeremiah Essig and Katelyn Sherland for launching the laboratory research that instigated this paper. HRT, BTH, NNH, and the rest of the team for an unforgettable research experience.

- Dr. Shafiqur Rahman, for his willingness to correspond with me personally to clarify many aspects of IgY preparation and administration to pediatric patients.

- My parents, Beth and Trevor Brown, for their example and the countless hours of teaching, discipline, love, personal counseling, and self-less sacrifice that comprised my training as a child and have continued to this day. I am in most areas the woman I am today because of you both and the work of the Holy Spirit in my life.

- Caleb, Charis, Nana, Grandma & Grandpa, you have all contributed immeasurably to who I am as well.

- The homeschooling parents who sparked my love for learning, science, and a search for Truth in those formative years.