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(71) Applicant: BHARAT BIOTECH INTERNATIONAL LIMITED [IN/IN]; Genome Valley, Turkapally, Shameerpet, Hyderabad 500078 (IN).

(72) Inventors: VADREVU, Krishna Mohan; Bharat Biotech International Limited, Genome Valley, Turkapally, Shameerpet, Hyderabad 500078 (IN). PRASAD, Sai Devarajulu; Bharat Biotech International Limited, Genome Valley, Turkapally, Shameerpet, Hyderabad 500078 (IN). ELLA, Krishna Murthy; Bharat Biotech International Limited, Genome Valley, Turkapally, Shameerpet, Hyderabad 500078 (IN).

(74) Agent: HASAN, Afzal et al.; Hasan and Singh, Flat No. 04, Sree Nilayam Apartment, Plot No. 12, Camelot Layout (Near Chirec Public School), Kondapur, Hyderabad 500084 (IN).

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(54) Title: A BUFFER FREE, ACID STABLE LOW DOSE VOLUME ROTAVIRUS VACCINE

(57) Abstract: A buffer free, acid stable, low dose volume rotavirus vaccine is disclosed. The vaccine is available in dose volume of less than 1 ml per dose for oral administration and it is without any buffer. The vaccine also does not require pre or post administration of any antacid at the time of oral administration of the vaccine to the subject to neutralize the stomach acid. The vaccine exemplifies nominal drop in vaccine titer at pH 2-4 for a time span of 30 minutes. The vaccine is stable at -20°C for at least 60 months.



A BUFFER FREE, ACID STABLE LOW DOSE VOLUME ROTAVIRUS VACCINE

FIELD OF THE INVENTION

5 The invention relates to the field of rotavirus vaccines. More particularly, this invention relates to the field of rotavirus vaccines which are buffer-free or do not require any antacid pre, during or post administration of the rotavirus vaccine. This invention also relates to the field of amount of dose volume per unit vaccine, particularly rotavirus vaccines.

10

BACKGROUND OF THE INVENTION

It is well known that Rotaviruses are the leading cause of severe Gastroenteritis in infants and in children worldwide. Even improvements in hygiene, water quality and sanitation that may generally control many bacteriae and parasites which are
15 responsible for causing other types of diarrhea do not adequately prevent the spread of rotavirus. Therefore, vaccination is the only strategy capable of significantly reducing the disease burden caused by rotavirus infections in underdeveloped and developing countries across the globe. Vaccination is more significant for the countries which lack the required economic support and infrastructure to address the rotavirus infections in
20 the infected patients. Till date, there have been at least four commercialized rotavirus vaccines in the world, the first among those being Rotashield® supplied by Wyeth Holdings, in the year 1999. This was a live, oral, tetravalent rotavirus vaccine produced using 1 rhesus monkey rotavirus, 3 rhesus-human reassortant viruses vaccine licensed in the United States. However, it was withdrawn from the market after 14 months
25 because of its association with adverse affects of intussusception.

The Rotashield® comprised a composition containing, sucrose, monosodium glutamate, pottasium monophosphate, potassium diphophate, fetal bovine serum and neomycin sulphate in amphotericin medium. Corresponding patent publication on
30 rotavirus vaccine compositions WO2000006196A2, teaches that the vaccine compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, tri-ethanolamine oleate, citrate-
35 bicarbonate, or the like. When the composition is administered orally it may also be

necessary to provide the individual a buffer solution to partially neutralize stomach acid and protect the reassortant rotavirus while passing to the intestine. Buffer solutions appropriate for this use include sodium bicarbonate, citrate bicarbonate, or the like.

- 5 RotaTeq®, is a pentavalent human-bovine (WC3) reassortant live-attenuated, oral vaccine manufactured by Merck & Co., comprising a Bovine rotavirus strain WC3, P7[5]G6 involving 5 reassortants; out of which 4 reassortants with the VP7 gene from G1, G2, G3, or G4 and 1 reassortant with the VP4 P1A[8] gene from the human rotavirus parent strain with the remainder of the genes from the WC3 bovine rotavirus
- 10 parent. The vaccine formulation (corresponding patent publications of Merck viz. WO1998/13065, WO2001/08495 and WO2002/011540) contains certain stabilizers in a tissue culture media along with the presence of buffers in the formulation itself, the said buffer being sodium citrate and sodium phosphate. The carboxylate of the patented claims are being used and acts as a buffer to make the rotavirus antigen withstand the
- 15 stomach acidity while the virus antigen gets administered to the infant's stomach.

Further, Rotarix® is a monovalent vaccine, comprising a passaged Human rotavirus strain RIX4414 derived from the 89-12 strain. Rotarix® (produced by GlaxoSmithKline Biologicals), is a live vaccine that contains the attenuated

20 monovalent G1, P[8] human rotavirus strain. The vaccine is available in lyophilized form mixed with stabilizers, to be reconstituted with a calcium carbonate based buffer diluent prior to vaccine administration into the subjects.

Rotarix was prepared by reconstituting the lyophilized active ingredient (RIX4414 strain + excipients) with 1.0 ml of GSK's CaCO₃ buffer contained in a pre-filled

25 syringe. After injecting the buffer into the vial containing the lyophilized vaccine, the vial was shaken well to resuspend the vaccine. The entire volume of resuspended product was then withdrawn into the same syringe, the needle (or transfer device) of the syringe was discarded, and the product was administered promptly as a single oral

30 dose. Alternatively, reconstitution of the commercial lyophilized vaccine will follow the same procedures, using an oral applicator filled with buffer and a transfer adapter instead of a syringe and needle. The reconstituted vaccine should appear milky white in appearance. It should be inspected visually for particulate matter and/or discoloration, and not be administered if either of these conditions exists. Rotarix is to be

35 administered orally. The infant should be seated in a reclining position, and the entire

content of the oral applicator containing the reconstituted vaccine should be administered on the inside of the cheek. Rotarix is not to be administered by injection. Hence, it is apparent that, reconstitution of buffer diluent contains so many steps, right from ensuring that the reconstitution has been properly done to the point of administration of the vaccine. All these factors definitely, cause decrease in vaccine compliance, amounting in partial protection from rotavirus infection rather than achieving a complete protection.

Corresponding patent applications from GSK on rotavirus vaccine formulations WO2001/012797, WO2005/02133, WO2006/087205, which involves a live attenuated rotavirus strain P43 serially passaged with specific mutations also supplemented with an antacid buffer for reconstitution. The said rotavirus strain G protein (VP4 and VP7) capable to induce immune response to atleast 4 other rotavirus strains to G1 and atleast one of G2 to G14 serotypes.

Other patented rotavirus vaccine compositions with stabilizers and buffers by Bharat Biotech International Limited with an Indian asymptomatic strain 116E isolated from a human child have also been disclosed. These require separate administration of an antacid buffer to the vaccine recipient prior to receiving the vaccine to increase the pH of the stomach, wherein the said antacid buffer is a citrate-phosphate buffer, in order to neutralize the stomach acidity at the time of administration of the rotavirus vaccine antigen. Patent Publication WO2013160913, provides rotavirus vaccine compositions comprising rotavirus antigens, stabilizers and certain novel buffer combinations. The buffers in the invention are pre-mixed in the rotavirus vaccine compositions to neutralize the high acidic pH of the stomach without, requiring separate administration of an antacid before vaccine administration.

Thus it is evident that different rotavirus vaccines have been developed using different strains, both human and animal reassortant types. There are a few important differences in characteristics of these strains, typical ones, as listed below.

Rotavirus vaccines with human strains	Animal reassortant rotavirus vaccines
Natural infection offers protection against subsequent rotavirus disease.	Animal rotaviruses are naturally attenuated strains, which lowers replication in humans.

Human viruses have high replication capacity in the host, and therefore attenuated by multiple tissue culture passage in multiple hosts. Since, these are human strains, a vaccine titer of 10^4 FFU/0.5 ml is enough to induce required immunogenicity against rotavirus infections in humans.	Rotavirus vaccines with animal reassortant strains require very high vaccine titers to be able to induce the required immune response.
Broad immunity is acquired through various immune effector mechanisms.	Expectation that neutralizing antibody in the gut lumen is required. Immunity obtained is not high as vaccines with human strains.
Heterotypic protection is gained through broad immune response. This means, it offers cross protection across different strains.	Reassortant vaccine constructs to include the common human rotavirus antigens.

Table 1: Differences between Human Monovalent and Animal reassortant vaccines

RATIONALE BEHIND THE PRESENT INVENTION

Thus it is known that rotavirus is highly sensitive to acidic environments (Estes, M., Graham, D., Smith, E. and Gerba, C. (1979). Rotavirus Stability and Inactivation. *Journal of General Virology*, 43(2), pp.403-409). It has also been reported that infantile gastroenteritis virus becomes unstable and there is a collapse of their outer shell and capsomeres when subjected to environment having pH less than 3.0 (Palmer, E., Martin, M. and Murphy, F. (1977). Morphology and Stability of Infantile Gastroenteritis Virus: Comparison with Reovirus and Bluetongue Virus. *Journal of General Virology*, 35(3), pp.403-414). It has also been reported that many strains of rotavirus have been inactivated within a minute when exposed to environment having pH 2, and similar results were obtained with human gastric juice having pH 1.8 (Weiss, C. and Clark, H. (1985). Rapid Inactivation of Rotaviruses by Exposure to Acid Buffer or Acidic Gastric Juice. *Journal of General Virology*, 66(12), pp.2725-2730). It has further been reported that human serotype 1 rotavirus is completely inactivated when subjected to an environment of pH 2.5 (Meng, Z., Birch, C., Heath, R. and Gust, I. (1987). Physicochemical Stability and Inactivation of Human and Simian Rotaviruses. *Applied and Environmental Microbiology*, 53(4), pp.727-730). Rotavirus, in general, is known to be highly unstable when subjected to acidic environments and rapidly gets inactivated. Inactivation rates of three bovine and several primate-origin rotaviruses were determined during exposure to acid buffers at pH 2.0, pH 3.0 or pH 4.0. Each rotavirus was inactivated at pH 2.0 (the acidity most resembling the normal fasting

stomach) very rapidly, with half-lives for infectivity determined to be 1 min or less. Each rotavirus was inactivated at a much slower rate at pH 3.0; inactivation at pH 4.0 was minimal. Some differences in acid resistance between different rotavirus strains were detected. Although these determinations were performed at room temperature
5 (23°C), experiments at diverse temperatures indicated an even more rapid rate of viral inactivation by acid at normal body temperature (37°C). Studies of rotavirus exposed to natural human gastric juice at pH 1.8 or pH 2.1, revealed a rate of virus inactivation similar to that observed with glycine buffer of identical pH. (Ref: Geigy Scientific Tables, volume 1, 1981, page 126).

10 Rotavirus inactivation takes place between pH 2.0 to pH 3.0 but at pH 4.0 no or minimal inactivation takes place. Since, the stomach contains highly gastric acid environment, it has been imperative to add buffers into the rotavirus vaccine formulations till date, Buffers in rotavirus formulations aid in retaining a constant pH
15 of the rotavirus vaccine formulations thus enabling antacid or buffering capacity of the vaccine formulations. The antacid capacity (also termed as acid neutralization capacity) of the rotavirus vaccines have been measured by Baby Rossett Rice Assays in the GSK's patents. The acid neutralization capacity of a given formulation is defined as the time measured to maintain the pH above 4.0 and is evaluated by Baby Rossette Rice
20 assay. Baby Rosset assay reportedly is alleged to simulate the gastric environment of the human stomach. The latest patent by GSK US8821891B2 advocates for lower concentration of phosphates, and with an increased concentration of a carboxylate preferably adipate ranging from 100 mM to 1M, preferably 400 mM to 700 mM in a dose volume as low as 1.5 ml per human dose of rotavirus vaccine. Adipate acts as a
25 buffer capable to withstand the high stomach acidity (pH 1 to 2). The capacity to withstand human stomach acidity is measured by Baby Rosset Rice Assay, having a value of 8 to 23 minutes which is said to be capable and good enough to avoid getting inactivation of the rotavirus antigen in the vaccine formulation after administration of the rotavirus vaccine into the human infant.

30 Thus, to effectively, administer a rotavirus vaccine which is capable to elicit immunogenic response sufficient enough to confer protection against rotavirus infections, at least 1.5 to 2 ml of the rotavirus vaccine or reconstituted vaccine formulation per human dose for oral administration is needed to effectively allow the

rotavirus antigen to pass through the highly acidic environment of the stomach. Therefore, by the above descriptions of the status of the present nature of the rotavirus vaccines available till date, it would also be easily understood and acknowledged by the persons having skilled in the art, that rotavirus antigen is a highly acid labile antigen, and administration of rotavirus vaccine always requires an antacid or buffer component thereby increasing the required quantity (in terms of dosage in volumes per unit vaccine) of vaccine administration.

It is also a fact that the rotavirus antigen is a highly unstable virus and considering the situation that live attenuated rotavirus vaccine antigen would be more unstable; therefore the rotavirus antigen are being supplemented with respective proprietary vaccine stabilizers to stabilize the vaccine formulations, which add up to the amount of the vaccine dose in terms of volume per unit vaccine. Furthermore, inclusion of buffers or antacids or requiring reconstitution of the vaccine with a buffer based vaccine diluent increases the amount of vaccine dose in terms of volume per unit vaccine. Hence, providing a rotavirus vaccine without a buffer/antacid will definitely reduce the dosage volume per unit vaccine. At the same time, it is imperative that such a rotavirus vaccine without a buffer should not be compromising with the efficacy as well as stability of rotavirus vaccine.

Since, the subject of the rotavirus vaccine recipient is always an infant within the age group between 6 weeks to 6 months of age, there is every possibility that a considerable amount of rotavirus vaccine formulation delivered into the mouth of the infant gets spilled out. This creates a chance of partial administration of the vaccine thereby reducing vaccine compliance and not protecting the disease burden inspite of receiving the vaccine. Therefore, it is always desirable that the amount of dosage in terms of volume per unit rotavirus vaccine is reduced as much as possible without compromising the efficacy of the vaccine formulation. Currently the least dosage volume disclosed in the state of the art for rotavirus vaccine range from 1.5 ml-2.5 ml. The vaccines contain vaccine stabilizers and vaccine buffers adding upto the total vaccine dose volume. So, decrease in dosage volume per unit rotavirus vaccine having the same capacity to raise required immunogenicity for prophylaxis against rotavirus infections in infants will decrease the possibility of spilling out of the vaccine formulation administered to the infant. It would therefore be highly non-obvious to

make available a low dose rotavirus vaccine formulation upto 1ml, preferably only 0.8ml, or most preferably 0.5 ml only per human dose without a buffer which is equally or rather, more effective than the rotavirus vaccine(s) made available with buffers or antacids having high dosage volume per unit vaccine.

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The prior art discloses all rotavirus vaccine formulations all of which are to be stabilized in liquid formulations at refrigerated temperatures between $5\pm 3^{\circ}\text{C}$ or as lyophilized powders which require reconstitution of the vaccine prior to the administration of the vaccine with a suitable diluent. It has been already discussed that, reconstitution of the vaccine for lyophilized powder require specific skill, and care. Further, separate manufacturing requirements for diluents along with the vaccine doubles the vaccine manufacturing costs as well as transportation costs. Deficiency in skill, care and appropriate infrastructure for warehouse requirements adds up to the problem with lyophilized rotavirus vaccine supply. For liquid vaccines at $5\pm 3^{\circ}\text{C}$, the shelf life of the vaccines are limited to only upto 2 years. There is also an associated titer loss with vaccines made available at $2-8^{\circ}\text{C}$, during storage and transportation which is undesirable. But, rotavirus vaccine formulations at -20°C , it has been found that there has been no transportation and storage loss at all. The chances of vaccine contamination is also reduced to much greater extent for vaccines at -20°C . Further, the vaccine supply facility at -20°C has been already established in almost 120 countries accross the World for polio vaccine supplies. Therefore, according to existing facilities for vaccine storage and vaccine supplies similar to polio vaccine supplies would greatly help in reducing the rotavirus disease burden in the World. It is desirable that alternative strategies be developed wherein the rotavirus vaccine stability be further increased, so that the vaccine may be made available to remotest corners of the globe. Therefore, novel rotavirus vaccine formulations are being disclosed in this patent application, wherein the rotavirus vaccine formulations are stable for atleast 5 years, being absolutely new and not stated in the current state of the art at all.

30 **OBJECTIVE OF THE PRESENT INVENTION**

The primary objective of the invention is to provide a buffer free and acid stable rotavirus vaccine.

One more primary objective of the invention is to provide a low dose volume upto 1 ml or even only 0.8 ml, preferably a 0.5ml low dose volume rotavirus vaccine.

35

Another objective of the invention is to provide a stable rotavirus vaccine composition with vaccine volume of as low as 0.5 ml per dose.

- 5 Another objective of the invention is to reduce spilling out of rotavirus vaccine and minimizing associated vaccine wastage by the vaccine recipient and increase vaccine compliance thereby.

10 Another objective of the invention is to make a rotavirus vaccine composition that does not require any specific dilution or reconstitution techniques prior to or during vaccine administration.

One more objective of the invention is to provide a rotavirus vaccine which is stable for at least 5 years at -20°C.

- 15 A further objective of the invention is provide a low dose volume rotavirus vaccine which is stable for 2 years at $5\pm 3^{\circ}\text{C}$, and 6 months at 25°C , and 1 week at 37°C .

20 One further objective of the invention is to provide a buffer free, low dose volume upto 1ml rotavirus vaccine composition which is stable in stomach at pH 2 to pH 4 for atleast 20 minutes.

25 Another objective of the invention is to provide a buffer free, low dose volume up to 1 ml rotavirus vaccine composition which is stable at acidic pH of 2-4 without any supplement of buffers or acid stabilizing agents.

A further objective of the invention is to provide a buffer free, acid stable, low dose volume rotavirus vaccine composition which does not require administration of any antacid or buffer, pre or post or during administration of the vaccine to the subject.

- 30 Alternatively, it is also an objective of the invention is to provide a low dose volume rotavirus vaccine of 0.5 ml along with the presence of a buffer, which is equally effective against rotavirus infections in humans.

35 Further objective of the invention is to provide a low dose volume rotavirus vaccine without a buffer, which is capable to generate either equivalent or greater immune

response than rotavirus vaccines which are supplemented with antacids or buffers in vaccine compositions.

SUMMARY OF THE INVENTION

5 According to one embodiment of the invention provides a vaccine composition wherein the loss in rotavirus vaccine titer is not more than 0.55 FFU/ per 0.5 ml between pH of 2-4 for a period of at least one hour.

10 In a further embodiment, the invention provides a vaccine composition wherein the loss in rotavirus vaccine titer is not more than 0.68 FFU/ per 0.5 ml between pH of 2-4 for a period of at least 20 minutes.

One more embodiment of the invention, provides various rotavirus vaccine composition at a dose volume ranging from 1 ml to 0.5 ml, wherein the vaccine
15 formulations are without any buffers or antacids, and exemplifies nominal drop in vaccine titer at pH 2 and pH for a time span of 30 minutes.

One another embodiment of the invention, provides acid neutralization capacities of a low dose volume rotavirus composition between 0.5 ml to 1 ml per unit vaccine in
20 coparison with rotavirus vaccine compositions in presence of buffers established through Baby Rosset Rice assays.

A further embodiment of the invention, provides a buffer free rotavirus vaccine of dose volume only 0.5 ml which is capable to generate either equivalent or greater immune
25 response in terms of four-fold seroconversion post vaccination similar to that of serconversion achieved with the vaccine formulation either in presence of buffers or administration of antacids.

In another embodiment, the invention provides a method of prophylaxis against
30 rotaviral diarrhea in humans, the method comprising steps of administering an effective amount of a stabilized rotavirus vaccine formulation comprising rotavirus strain 116E, combination of sugars selected from sucrose, lactose and trehalose, lactalbumin hydrolysate, recombinant human serum albumin, without the presence of any buffer formulation in the said rotavirus vaccine formulation.

35 One more embodiment of the invention provides for stable rotavirus vaccine

compositions, which are stable at -20°C for at least 5 years.

A further embodiment of the invention provides stable rotavirus vaccine compositions at 2-8°C, 25 °C and 37°C for 2 years, 6 months and 3 weeks respectively.

5

DETAILED DESCRIPTION OF THE INVENTION

Embodiment 1: Buffered Rotavirus Formulations and Gastric acidity

It has been mentioned above in the background of the invention that, usage of buffers is
10 a common practice rotavirus vaccine formulation strategies, that has being undertaken
by various rotavirus vaccine manufacturers till date, and hence the Rota vaccine
comprising the 116E strain at -20°C formulation (SPG and DMEM) has been tested
with citrate-bicarbonate buffer, and normal saline conditions initially.

15 Bharat Biotech International Limited obtained the human rotavirus strains 116E from
National Institute of Health under a Material Transfer Agreement with National
Institute of Allergy and Infectious Diseases (NIAID), NIH, Bethesda, USA. The
complete genomic sequence of rotavirus strains 116E is already reported in literature.

20 Three Rotavirus vaccine formulations with rotavirus 116E stored at -20°C were taken
in 3 groups (Group I, II and III) as detailed below. The rotavirus vaccine compositions
in all the three groups below comprise of live attenuated Rotavirus antigen of the strain
116E dissolved in 10% SPG (sucrose, potassium dihydrogen phosphate, and
dipotassium hydrogen phosphate) and Dulbecco's Minimum Essential Medium. The
25 said vaccine formulations were treated separately as detailed below:

Group I: 0.5 ml of Rotavirus 116 E formulation A at -20°C.

Group II: 2 ml (equivalent 4 human doses of 0.5ml per human dose) of Rotavirus 116 E
30 formulation A mixed with 5 ml of Normal Saline.

Group III: 0.570 ml of Rotavirus 116 E formulation A + mix of [1.42ml Citrate -
Bicarbonate buffer + 8 ml of 34.8 mEq HCl].

35 As given above, Group I includes the rotavirus vaccine formulation at -20°C wherein
no buffer or saline was added to the vaccine formulation and the vaccine titer was
measured for next 120 minutes. IN Group II, 2 ml of rotavirus 116E vaccine

formulation (stored at -20°C) was taken and added with 5 ml of normal saline, and vaccine titer was measured at a target titer of 10^6 FFU/ml upto 120 minutes. In Group III, 0.57 ml of rotavirus 116E vaccine formulation (stored at -20°C) was taken and added with 1.42 ml of Citrate Bicarbonate Buffer and 8 ml of 34.8 mEq Hydrochloric acid.

Group#	0 mins in FFU/0.5 ml	60mins in FFU/ml	120mins in FFU/ml	Remarks
Group I	$10^{6.35}$	$10^{6.17}$	$10^{6.32}$	Highly stable
Group II	$10^{5.76}$	$10^{5.84}$	$10^{5.69}$	Fairly stable
Group III	$10^{4.61}$	$10^{5.30}$	$10^{5.11}$	Fairly stable

Table 2: Rotavirus vaccine formulation (at storage temperature: -20°C) vaccine titers in presence of buffers at 0 minute, 60 minutes and 120 minutes.

- Therefore, the above table establishes that both for exposing the virus to normal saline conditions or in presence of buffer solutions, the rotavirus antigen titer is stable, and therefore the vaccine is stable.

Embodiment 2: Immunoperoxidase Assay of Rotavirus Vaccine Formulations at different pHs.

- Example 1:** Immunoperoxidase Assay was done after subjecting the rotavirus vaccine formulations directly to the acidic environment by direct addition of the 34.8 mEq of Hydrochloric Acid (HCl) to the rotavirus vaccine formulation. The pH of the vaccine formulations were directly brought down from pH 7 to various lower pH values of pH 2, and pH 4 by direct addition of acid, and subsequently the vaccine antigen titer was measured at the given lower pH values of pH 2, and pH 4. Following this, the vaccine titer was measured for upto 1 hour at various lower pH of 2 and 4. Following observations were made. The reaction mixture was studied at various time points and various pH levels. Details are shown below in the table below. The said rotavirus vaccine formulations (at storage temperature -20°C) contains live attenuated rotavirus antigen 116E, 10% SPG (sodium phosphate glutamate) dissolved in Dulbecco's Minimum Essential Medium.

Sample details	Titer in FFU/0.5 mL dose					Remarks
	Actual titer at 0 minutes	Corrected Titer with Dilution Factor at 0 minutes	Actual titer after 1 hour	Corrected Titer with Dilution Factor after 1 hour	Log loss after 1 hour.	
0.5 ml Rotavirus 116E vaccine formulation at pH 7.4	$10^{4.95}$	NA	$10^{4.94}$	$10^{4.94}$	-	-
0.5 ml Rotavirus 116E vaccine formulation + 34.8 mEq HCl at pH 4.0	$10^{3.58}$	$10^{5.05}$	$10^{3.58}$	$10^{5.05}$	Nil	No effect of pH
0.5 ml Rotavirus 116E vaccine formulation + 34.8 mEq HCl at pH 2.0	$10^{3.54}$	$10^{5.01}$	$10^{2.93}$	$10^{4.40}$	0.55	Marginal effect of pH

Table 3: Immunoperoxidase Assay results at a target titer of $10^{5.0}$ FFU/0.5 ml dose at 0 minutes and 60 minutes by direct addition of acid to rotavirus vaccine formulations (storage temperature: -20°C).

- 5 Note: Calculated reduction in Infectivity Titer value due to dilution = $10^{1.47}$ FFU. Therefore actual vaccine titer after dilution is $10^{2.93}$ at pH 2. All the samples were titrated in triplicate by Immunoperoxidase Assay. The oral rotavirus vaccine (target titer $10^{5.0}$ FFU / 0.5 mL) was stable at pH 4.0. Target Titer means, the minimum concentration of the rotavirus 116E antigen in the vaccine vial intended to be attained
- 10 in the given vial. The original titer at neutral pH of the vaccine is $10^{4.95}$ FFU/0.5 ml at 0 minutes. The vaccine titer at pH 2 at 0 minutes is $10^{3.54}$ FFU/0.5 ml and the titer dropped to $10^{2.93}$ FFU/0.5 ml within 1 hour. So, after adding the dilution factor value of 1.47 due to addition of the acid, with the actual titer value ($10^{2.93}$ FFU/0.5ml), the corrected vaccine titer at pH 2 after 1 hour from addition of the acid is $10^{4.40}$ as
- 15 compared to the original vaccine titer of $10^{4.95}$ at pH 7 at 0 minutes. Therefore, the vaccine experienced a total log loss in vaccine titer of 0.55 only at pH 2.0 within a period of 1 hour. This establishes that, even at lower pH value of 2, that is to say in a specific acidic environment as it appears in the human stomach, the rotavirus vaccine formulation is capable to sustain its vaccine titer at a value of $10^{4.40}$ FFU/0.5 ml. At

such vaccine titer, the vaccine is capable to raise sufficient immune response against subsequent rotavirus infections. In a clinical trial carried out with Rota 116E vaccine at a titre of $10^{4.0}$, the vaccine yielded a 4 fold sero-conversion around 62%, which is quite encouraging (Nita Bhandari et al, J Infect Dis 2009, 200, 421-9).

5

Example 2: The same experiment has been repeated with a different vaccine (at storage temperature -20°C), wherein the target titer is $10^{6.0}$ FFU/ 0.5 ml (dose) Calculated titer values along with dilution factor are provided in the following table at various pH values at 0 minutes and after 1 hour. The said rotavirus vaccine formulations (at storage temperature -20°C) contains live attenuated rotavirus antigen 116E, 10% SPG dissolved in DMEM.

10

Vaccine Sample Details	pH	'Corrected Titer with Dilution Factor at 0' Hr in FFU/0.5 ml	Corrected Titer with Dilution Factor after 1 hour in FFU/0.5ml	Log loss after 1 hour	Remarks
0.5 ml Rotavirus 116E vaccine formulation	7.2	$10^{5.35}$	$10^{5.30}$	0.05 (no loss)	No effect of pH
0.5 ml Rotavirus 116E vaccine formulation + 34.8 mEq HCl	4.0	$10^{5.33}$	$10^{5.09}$	0.24 (marginal loss)	No effect of pH
0.5 ml Rotavirus 116E vaccine formulation + 34.8 mEq HCl	2.0	$10^{4.89}$	$10^{4.21}$	0.68 (marginal loss)	Marginal effect of pH

Table 4 : Immunoperoxidase Assay results at a target titer of $10^{6.0}$ FFU/0.5 ml dose at 0 minutes and 60 minutes by direct addition of acid to rotavirus vaccine formulations (storage temperature: -20°C).

15

Conclusion: There was only loss in 0.24 log loss in vaccine titer in vaccine samples at pH 4.0, which is considered to be no effect on pH in practical circumstances kept for 1 hour. At pH 2.0, there was a 0.46 Log titer loss in the titer at 0 hour and a loss of 0.68 log₁₀ FFU at 1 hour. This is said to be having very marginal effect on pH on the vaccine titer values at such an acidic lower pH value of 2.

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According to the above table, we see that vaccine titer of $10^{4.89}$ FFU/0.5ml at pH 2 is retained till a titer value of $10^{4.21}$ FFU/0.5 ml; a log titer of 4.89-4.21 is good enough for replication of the rotavirus in the gut of an infant stomach at an age of 6 weeks to 2 years. The stomach acidity of an infant is also pH 2, representing a simulated gastric environment. The vaccine is still able to survive having a titer of 4.89 Log 10 FFU/0.5 ml, which is enough to cross the stomach and then get established into the intestinal villi and for further replication of rotavirus, responsible for generating immune response in the infant, thereby conferring protection and prevention of rotaviral gastroenteritis. Therefore, the vaccine was found to be stable at acidic pH of 2 for at least 1 hour duration without having any considerable loss in vaccine titer at all.

Embodiment 3: In vitro Experiment to demonstrate that Rotavirus 116E vaccine does not require antacid:

Apart from the conclusions from the Clinical trial results that follow later in the patent specification, it was also proved in vitro simulating similar conditions of stomach acidity administering the vaccine directly without buffer.

Example 3.1: 1 mL of vaccine (formulation 3A) containing rotavirus 116E, combination of sucrose (50%), lactose (0.5%) and trehalose (0.5%), lactalbumin hydrolysate (1 %), human serum albumin (0.4%) , was taken and added with 1.25 mL of 0.1 N HCl and mixed well. Sample was taken and tested for virus content at various pH levels at different time intervals. The results are shown in the table 5.1 below.

S.No	Sample details	Virus content (FFU/mL)
1	pH 2.0-0 minutes	5.72
2	pH 2.0- 10 minutes	4.78
3	pH 2.0- 30 minutes	3.84
4	pH 3.0-0 minutes	5.96
5	pH 3.0- 10 minutes	5.96
6	pH 3.0- 30 minutes	5.91
7	Control	6.10

Table 5.1: Immunoperoxidase assay of 1ml vaccine with rotavirus 116E (formulation 3A)

Example 3.2: 0.8 mL of vaccine containing rotavirus 116E, combination of sucrose (40%), and trehalose (0.5%), lactalbumin hydrolysate (20%), polyvinyl pyrrolidone (0.5%) (formulation 3B) was taken and added with 1.25 mL of 0.1 N HCl and mixed well. Sample was taken and tested for virus content at various pH levels at different time intervals. The results are shown in the table 5.2 below.

S.No	Sample details	Virus content (FFU/mL)	Virus content (FFU/0.8mL)
1	pH 2.0-0 minutes	4.71	4.62
2	pH 2.0- 10 minutes	4.69	4.60
3	pH 2.0- 30 minutes	4.41	4.32
4	pH 3.0-0 minutes	5.97	5.88
5	pH 3.0- 10 minutes	5.94	5.85
6	pH 3.0- 30 minutes	5.93	5.84
7	Control	6.10	

Table 5.2: Immunoperoxidase assay of 0.8 ml vaccine with rotavirus 116E (formulation 3B)

Example 3.3: 0.5 mL of Rotavirus 116E vaccine containing combination of sucrose (60%), and trehalose (0.5%), soy protein (20%), pyridoxine-HCL (0.1%), polyvinyl pyrrolidone (0.25%) (formulation 3C) was taken and added with 1.25 mL of 0.1 N HCl and mixed well. Sample was taken and tested for virus content at various pH levels at different time intervals. The results are shown in the table 5.3 below.

S.No	Sample details	Virus content (FFU/0.5 mL)
1	pH 2.0-0 minutes	4.29
2	pH 2.0- 10 minutes	4.21
3	pH 2.0- 30 minutes	3.39
4	pH 3.0-0 minutes	5.20
5	pH 3.0- 10 minutes	5.17
6	pH 3.0- 30 minutes	5.08
7	Control	5.80

Table 5.3: Immunoperoxidase assay of 0.8 ml vaccine with rotavirus 116E (formulation 3C)

Example 3.4: 0.5 mL of Rotavirus 116E vaccine containing 10% SPG dissolved in DMEM (formulation 3D) was taken and added with 1.25 mL of 0.1 N HCl and mixed well. Sample was taken and tested for virus content at various pH levels at different time intervals. The results are shown in the table 5.4 below.

S.No	Sample details	Virus content (FFU/0.5 mL)
1	pH 2.0-0 minutes	4.78
2	pH 2.0- 10 minutes	4.48
3	pH 2.0- 30 minutes	4.29
4	pH 3.0-0 minutes	5.22
5	pH 3.0- 10 minutes	5.17
6	pH 3.0- 30 minutes	4.70
7	Control	5.80

5

Table 5.4: Immunoperoxidase assay of 0.8 ml vaccine with rotavirus 116E (formulation 3D)

Example 3.5: 0.5 mL of Rotavirus 116E vaccine containing sucrose (40%), trehalose (0.5%), lactose (5%), rHSA (0.5%), LAH (1%) and mixed buffer (ammonium acetate + ammonium bicarbonate + diammonium orthophosphate) (formulation 3D) was taken and added with 1.25 mL of 0.1 N HCl and mixed well. Sample was taken and tested for virus content at various pH levels at different time intervals. The results are shown in the table 5.5 below.

10

S.No	Sample details	Virus content (FFU/0.5 mL)
1	pH 2.0-0 minutes	4.77
2	pH 2.0- 10 minutes	4.65
3	pH 2.0- 30 minutes	4.39
4	pH 3.0-0 minutes	5.21
5	pH 3.0- 10 minutes	5.19
6	pH 3.0- 30 minutes	4.87
7	Control	5.80

Table 5.5: Immunoperoxidase assay of 0.8 ml vaccine with rotavirus 116E (formulation 3D)

Conclusions: From the above tables it is evident that at pH 2.0 for 30 minutes also any Rotavirus 116E vaccine formulations are able to withstand in absence of any buffer or any antacid.

5 Embodiment 4: Baby Rossett Rice Analysis

BRR assay is the validated procedure for acid neutralization capacity of rotavirus vaccine formulations. So selected vaccine formulations containing Citrate Phosphate Buffer and Citrate Bicarbonate Buffer are evaluated for Acid Neutralization Capacity using this assay. Invitro experiments were conducted under simulated conditions of infantile stomach using Baby Rosette Rice Assay (BRR). Various formulations with various combinations of buffering agents were selected for the acid neutralization experiments and the results are given in the table below at various pH values wherein the conditions were simulated as infant stomach. The vaccine formulations were diluted with water for injection upto 10 ml, then added 4 ml of 0.1 N HCl, then 0.5 ml of 0.1 N HCl added per minute until pH reaches 4.0. ANC is defined as the time in minutes taken to maintain the pH above 4.0.

Vaccine Formulation 4A	Rotavirus antigen 116E target titer of 10^6 FFU/0.5ml; 10% SPG, and DMEM (quantity sufficient)
Vaccine Formulation 4B	Rotavirus antigen 116E target titer of 10^6 FFU/0.5ml; 10% SPG, and DMEM (quantity sufficient) added to 2.5 ml of Citrate Bicarbonate Buffer
Vaccine Formulation 4C	Rotavirus antigen 116E target titer of 10^6 FFU/0.5ml, combination of atleast 2 Sugars (sucrose 50% and trehalose 0.5%) and Lactalbumin hydrolysate 0.5%. (Sample 8 below).
Vaccine Formulation 4D	Rotavirus antigen 116E target titer of 10^6 FFU/0.5ml, combination of atleast 2 sugars (Sucrose-40%, Trehalose-0.5%), 0.5% lactalbumin hydrolysate, 0.35% human serum albumin and 1.1M Phosphate Buffer.

Table 6: Details of Vaccine Formulations:

Following observations (Table 7) were made for Baby Rosset Rice assay performed with the vaccine formulations 4A to 4D listed above.

Vaccine Formulations	Initial pH	pH after addition of Acid	Volume of 0.1N HCl added	Baby Rosset Rice Assay Values	Initial Vaccine Titers in FFU/0.5 ml	Vaccine Titers in FFU/0.5 ml After 1 hour
Vaccine formulation 4A at -20°C without buffer of 0.5 mL dose.	7.48	1.95	4.0 mL	BRR of less than 0 minutes (immediate drop in pH)	$10^{4.83}$	$10^{3.83}$
Vaccine formulation 4B at -20°C with 2.5 ml of citrate bicarbonate buffer of 0.5 mL dose.	8.35	4.22	10.0 mL	BRR of 12 minutes	$10^{6.17}$	$10^{6.15}$
Vaccine formulation 4C at $5\pm 3^\circ\text{C}$ without buffer of 0.5 mL dose.	7.28	3.98	5.0 mL	BRR of 2 minutes	$10^{5.30}$	$10^{5.02}$
Vaccine formulation 4D at $5\pm 3^\circ\text{C}$ with buffer of 1.5 mL or 2 mL dose.	6.75	4.33	10.5 mL	BRR of approximately 13 minutes	$10^{6.12}$	$10^{6.11}$

Table 7: Baby Rosset Rice analysis in comparison to rotavirus vaccine formulations with and without buffer.

- 5 Conclusion: Therefore from the above table we find that, even if for the vaccine formulation 4A above which shows immediate drop from pH 7.48 to pH 1.95, the vaccine titer is retained from $10^{4.83}$ FFU/0.5 ml and $10^{3.83}$ FFU/0.5 ml. Same way, the vaccine titer is also retained in case of Vaccine formulation 4C between $10^{5.30}$ to $10^{5.02}$ FFU/0.5 ml where the pH drops from 7.28 to pH of 3.98 within 2 minutes.
- 10 Therefore, even if the BRR value is only 2 minutes, the vaccine titer is fairly retained i.e. $10^{5.02}$ FFU/0.5 ml. This much amount of vaccine titer is enough for generation of immune response and thereby conferring protection and prevention of rotaviral gastroenteritis in human infants.
- 15 **Embodiment 4: Clinical trial without Buffer for ROTAVAC® comprising rotavirus 116E strain.**

Hypothesis and Study Rationale

Natural transmission of rotavirus is assumed to occur via faecal-oral route. Rotaviruses in general are acid labile and it is believed that acidic environment affects the viability of the virus.

- 5 Given their acid-labile nature of rotaviruses, are so efficient in ubiquitously infecting mammals, most of which have gastric pH values around 2. One possibility is that the human infant stomach may be somewhat more permissive for survival of rotavirus than the adult stomach, as infant gastric pH levels tend to be approx. 3.2 compared with adults at approximately 1.0. This could account for the fact that 60 to 90% of reported
- 10 human rotavirus disease occurs in children below the age of 3 years.

Study Design

- In order to test this hypothesis a large multicenter randomized controlled trial was undertaken to evaluate and compare the vaccine immune response in subjects receiving
- 15 ROTAVAC with citrate bicarbonate buffer to those who receive ROTAVAC without the buffer.

Accordingly, the study included the following three treatment groups:

- Group I (received ROTAVAC[®] with 5 minutes prior administration of 2.5 ml of
- 20 buffer),
- Group II (received ROTAVAC[®] without buffer),
- Group III (received ROTAVAC[®] immediately mixed with 2.5 ml of buffer prior to administration).

- 25 The study investigator and safety assessors (study coordinators) were blinded to the treatment group assignment. Blinding was achieved by a study nurse administering the vaccine as per the allocated treatment group. All subjects received 3 doses of the vaccine given 4 weeks apart. Vaccine immune response was tested 4 weeks after the third vaccine dose (day 84) and compared to baseline (day 0). Immunogenicity was
- 30 tested in terms of Geometric Mean Titres (GMTs) of serum anti rotavirus IgA and seroconversion (fold rise in antibody levels from pre-vaccination to post vaccination) in the treatment groups.

Study Results and Conclusions

Post vaccination immune response was comparable with no statistically significant difference in the anti rotavirus IgA response between the treatment groups. Importantly, seroconversion and GMTs achieved in group II (ROTAVAC[®] administered without buffer) was similar to that in the other two treatment groups where ROTAVAC[®] was administered with buffer.

Post vaccination anti-rotavirus IgA GMTs in the group where ROTAVAC was administered without buffer was 20.7U/mL in comparison to 19.6U/mL and 19.2U/mL in the two groups which received antacid buffer 5 minutes prior to vaccine and simultaneously mixed with vaccine respectively (Table 8). The differences between the groups were not statistically significant ($p > 0.05$, Student's T-test).

Table 8: Geometric Mean Titres & 95% CI for results (day 0 & day 84)

Visit	Group I (N = 290)		Group II (N = 287)		Group III (N = 286)	
	GMT	95% CI	GMT	95% CI	GMT	95% CI
DAY0	10.5	(9.4, 11.7)	10.8	(9.7, 11.9)	10.2	(9.2, 11.3)
DAY84	19.6	(17.0, 22.7)	20.7	(17.9, 24.0)	19.2	(16.8, 22.1)

N = Number of Subjects evaluated in each group; GMT = Geometric Mean; 95 % CI (LL, UL) = Confidence Intervals (Lower Limit, Upper Limit)

Similarly, the four-fold seroconversion observed in the groups that received ROTAVAC[®] without antacid buffer and with buffer was similar at 29.2%, 24.5% and 25.1% respectively (Table 9). Further, the lower limit of the 95% confidence interval of the difference of seroconversion (2-fold) between the treatment groups was $> -10\%$, indicating non-inferiority in the immune response achieved with all three treatment regimens (table 10).

Table 9: Seroconversion and 95% CI (2, 3 or 4 fold change in antibody titres) between baseline and day 84 post-vaccination

Fold Change	Group I (N = 290)		Group II (N = 287)		Group III (N = 286)	
	%	95% CI	%	95% CI	%	95% CI
2	30.7	(25.7, 36.2)	35.2	(29.9, 40.9)	33.5	(28.3, 39.2)
3	27.6	(22.8, 33.1)	32.4	(27.3, 38.1)	31.8	(26.7, 37.4)
4	24.5	(19.8, 29.7)	29.2	(24.3, 34.8)	25.1	(20.5, 30.5)

N = Number of subjects evaluated in each group; (95 % CI) = Confidence Intervals (Lower Limit, Upper Limit)

Table 10: Difference in % 2-fold seroconversion at post vaccination in the treatment groups

% 2-fold Seroconversion post vaccination (day 84)						
	Group I (N = 290)		Group II (N = 287)		Group III (N = 286)	
	%	95 % CI	%	95 % CI	%	95 % CI
DAY 84	30.7	(25.7, 36.2)	35.2	(29.9, 40.9)	33.5	(28.3, 39.2)
Difference in % seroconversion with 95 % CI 2-Sided, Chi-Square Test)						
Group			% (95 % CI of difference)			
Group I Vs Group II			4.5 (-3.5, 12.5)			
Group II Vs Group III			1.6 (-6.5, 9.7)			
Group I Vs Group III			2.9 (-4.7, 10.5)			

The reactogenicity and safety with respect to solicited and unsolicited adverse events were comparable across the three groups with no statistically significant difference. ROTAVAC® vaccine was well tolerated in all three treatment groups that received the vaccine with or without the antacid buffer.

Embodiment 5: New Rotavirus 116E formulations

10 Stability data of ORV 116E liquid formulations at 37°C, 25°C and 2-8°C

The rotavirus vaccine formulations of a dose volume of 0.5 ml without bufer (samples 1 to 12, 14 to 16), and with mixed buffer at 0.5 ml dose volume (sample 13) have been examined for prolonged period of time ranging for a period upto 5 years, is provided below. The various formulation details is provided below along with their stabilites at 37°C for upto 4 weeks, 25°C for at least 6 months, and for 5±3 °C for upto 2years to 5 years. The studies establish that, the rotavirus vaccine formulations without buffer at dose volumes of 0.5 ml are also stable in nature for prolonged periods of time at refrigerated temperatures for at least 2 years. Rotavirus vaccine formulations are also found to be stable for a period of 5 years at refrigerated temperatures as well.

Sample 1 is formulated containing rotavirus 116E, Sucrose 50%, Lactose 0.5%, HSA 0.5% and Lactalbumin hydrolysate 0.05%.

Stability of Sample 1:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M	60M
5±3 °C	6.02	6.0			6.43		6.21	6.16	6.16	6.07
25°C	6.02	6.07	5.81	5.9	5.51	4.65				
37°C	6.02	5.32	2.63							

Sample 2 is formulated containing rotavirus 116E, Sucrose 50%, Trehalose 0.5%, HSA 0.5% and Lactalbumin hydrolysate 0.5%.

5

Stability of Sample 2:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M	60M
5±3 °C	6.09	6.14			6.19		6.07	6.10	6.05	6.01
25°C	6.09	6.02	6.06	5.63	5.51	2.48				
37°C	6.09	5.47	3.23							

Sample 3 is formulated containing rotavirus 116E, Sucrose 50%, Trehalose 1.0%, HSA 0.5% and Lactalbumin hydrolysate 0.5%.

10

Stability of Sample 3:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M	60M
5±3 °C	6.22	6.24			6.26		6.14	6.20	6.13	6.04
25°C	6.22	6.18	6.15	5.80	5.34	2.03				
37°C	6.22	5.35	3.22							

Sample 4 is formulated containing rotavirus 116E, Sucrose 40%, Lactose 0.5%, and Lactalbumin hydrolysate 1.0%.

15

Stability of Sample 4:

Temp	0 day	1M	2M	3M	6M	9M	12M	15M	24M	60M
5±3 °C	6.19	6.18			6.08		6.21	6.24	6.12	
25°C	6.19	5.66	5.10	4.8	3.01					
37°C	6.19	4.23	2.01							

Sample 5 is formulated containing rotavirus 116E, Sucrose 40%, Maltose 5.0% and Lactalbumin hydrolysate 1.0%.

Stability of Sample 5:

Temp	0 day	1M	2M	3M	6M	9M	12M	15M	24M	60M
5±3 °C	6.35	6.37			6.18		6.01	6.17	6.08	
25°C	6.35	5.89	5.11	4.77	2.43					
37°C	6.35	5.31	3.58							

Sample 6 is formulated containing rotavirus 116E, sucrose 40%, Trehalose 0.5% and
5 lactalbumin hydrolysate 1%.

Stability of Sample 6:

Temp	0 day	1M	2M	3M	6M	9M	12M	15M	24M	60M
5±3 °C	6.19	6.15			6.10		6.03	6.22	6.13	
25°C	6.19	5.87	5.43	5.12	3.25					
37°C	6.19	4.02	2.52							

Sample 7 is formulated containing rotavirus 116E, Sucrose 40%, Trehalose 1.0% and
10 Lactalbumin hydrolysate 1.0%.

Stability of Sample 7:

Temp	0 day	1M	2M	3M	6M	9M	12M	15M	24M	60M
5±3 °C	6.34	5.79			6.00		5.55	6.07	6.09	
25°C	6.34	6.07	5.81	5.9	5.51	4.65				
37°C	6.34	5.32	2.63							

Sample 8 is formulated containing rotavirus 116E, sucrose 50%, Trehalose 0.5%, and
15 lactalbumin hydrolysate 0.5%.

Stability of Sample 8:

Temp	0 day	1M	2M	3M	6M	15M	18M	24M	36M	60M
5±3 °C	6.34	6.34			6.33	6.31	6.24	6.14	6.08	

25°C	6.34	6.32	6.14	6.01	5.25					
37°C	6.34	5.11	4							

Sample 9 is formulated containing rotavirus 116E, Sucrose 70%, Trehalose 0.5%.

Stability of Sample 9:

Temp	0 day	1M	2M	3M	6M	15M	18M	24M	36M	60M
5±3 °C	6.38	6.31			6.23	6.44	6.35	6.26	6.18	
25°C	6.38	6.41	6.35	6.23	5.57					
37°C	6.38	5.07	4.01							

5

Sample 10 is formulated containing rotavirus 116E, Sucrose 50%, lactose 0.5%, Maltose 0.5%, HSA 0.5% and Lactalbumin hydrolysate 0.05%.

Stability of Sample 10:

Temp	0 day	1M	2M	3M	6M	15M	18M	24M	36M	60M
5±3 °C	6.25	6.15			6.03	6.34	6.08	6.12	6.11	6
25°C	6.25	6.14	6.27	6.12	5.88					
37°C	6.25	5.02	4.35							

10

Sample 11 is formulated containing rotavirus 116E, Sucrose 50%, Trehalose 0.5%, maltose 0.5%, HSA 0.5%.

Stability of Sample 11:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M	60M
5±3 °C	6.68	6.62	6.95	6.84	6.64	6.65	6.37	6.72	6.05	
25°C	6.68	6.50	6.20	6.04	5.32	4.47				
37°C	6.68	6.10	5.58	4.26						

15

Sample 12 is formulated containing rotavirus 116E, Sucrose 50%, Trehalose 0.5%, HSA 0.5% and Lactalbumin hydrolysate 0.05%.

Stability of Sample 12:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M	60M
5±3 °C	6.62	6.76	6.98	7.00	6.86	6.74	5.99	6.46	6.17	
25°C	6.62	6.46	6.42	5.99	5.13	3.25				
37°C	6.62	5.58	5.06							

- Sample 13 is formulated containing rotavirus 116E, sucrose (40%), trehalose (0.5%),
 5 lactose (5%), rHSA (0.5%), LAH (1%) and mixed buffer (ammonium acetate + ammonium bicarbonate + diammonium orthophosphate).

Stability of Sample 13:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M	60M
5±3 °C	6.61	6.59	6.58	6.60	6.53	6.54	6.18	6.01	5.83	
25°C	6.61	6.46	6.44	5.79	5.25	4.15				
37°C	6.61	5.56	5.01							

- 10 Stability of Sample 13 at -20°C:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M	60M
-20°C	6.61	6.59	6.59	6.56	6.54	6.52	6.21	6.11	6.13	6.09

- Sample 14, 15 and 16 are formulated with rotavirus 116E, 10% SPG dissolved in DMEM. The samples 14, 15, and 16 are kept at -20°C, and stability checked for 60 months. The vaccine formulations 13, 14, and 15 are found to be very stable at -20°C
 15 for a period of at least 5 years.

Vaccine sample	0 Day	3M	6M	9M	12M	24M	36M	48M	60M
Sample 14 (10 ^{4.0} FFU/0.5 mL)	4.25	4.01	4.08	4.15	4.25	4.02	4.18	4.12	4.08
Sample 15 (10 ^{5.0})	5.55	5.42	5.68	5.37	5.77	5.89	5.32	5.40	5.32

FFU/0.5 mL)									
Sample 16 (10^{6.0} FFU/0.5 mL)	6.12	6.12	6.06	6.33	6.36	6.27	6.15	6.11	6.05

Thus, it is established that various rotavirus vaccine compositions of 0.5 ml of dose volume are stable at -20°C for 5 years, 2-8°C for 2 years, 25°C for 6 months, and 37°C for 1 week. Irrespective of the vaccine formulation, the rotavirus vaccine at 0.5 ml to 1 ml dose volume is also found to be acid stable, as exemplified in embodiment 3 as well at a pH range of 2-4. Therefore, applicants wish to mention that irrespective of the vaccine composition details, any rotavirus vaccine comprising the live attenuated rotavirus 116E is capable to withstand the strong acidic environment in the human infant stomach and produce the required immune response against the rotavirus infections. The vaccine formulations presented in this patent application are only meant for the purposes of explaining the breadth and depth of the invention. It is impractical to provide all possible vaccine formulation details by the applicant, although the applicant has given numerous formulation strategies in Embodiment 3, Example 3 and Embodiment 5. Therefore, all possible obvious variations by a person skilled in the art for rotavirus vaccine formulation strategy comprising the rotavirus 116E strain are also covered under this invention. It is to be understood and accordingly construed that, the vaccine compositions which are not specifically included in this patent application comprising the live attenuated rotavirus 116E is also covered under the scope of this invention.

We Claim:

1. A buffer free, acid stable, rotavirus vaccine comprising rotavirus strain 116E, wherein the said vaccine formulation is capable of eliciting an immune response against rotavirus which is equal to or greater than the immune response generated by any conventional rotavirus vaccine with a buffer.
2. The rotavirus vaccine as claimed in claim 1, wherein the formulation is free of all kinds of buffers to protect the rotavirus antigen from stomach acidity.
3. The rotavirus vaccine as claimed in claim 1, wherein the said vaccine formulation is stable in stomach at pH 2 to pH 4 with no titre loss and is capable to withstand the stomach acidity in humans for a period of at least 20 minutes without any supplement of antacid or buffer.
4. The rotavirus vaccine as claimed in claim 1, wherein the vaccine does not require administration of any antacid or buffer, pre or post administration of the vaccine to the subject.
5. A rotavirus vaccine comprising rotavirus 116E, wherein the dose volume per unit vaccine is upto 1 ml only.
6. The rotavirus vaccine as claimed in claim 5, wherein the dose volume per unit vaccine is 0.8 ml per unit vaccine.
7. The rotavirus vaccine as claimed in claim 5, wherein the dose volume per unit vaccine is 0.5 ml per unit vaccine.
8. The rotavirus vaccine as claimed in claim 1 and 5, wherein the vaccine is stable at -20°C for at least 60 months.
9. The rotavirus vaccine as claimed in claim 1 and 5, wherein the vaccine is stable at 2-8°C for least 36 months, at 25 °C for 6 months and 37 °C for at least 4 weeks.
10. The rotavirus vaccine as claimed in claim 5 and 6, is with or without a buffer.
11. The vaccine formulation as claimed in claim 7, is with or without a buffer.
12. The rotavirus vaccine as claimed in claim 1 and 5, wherein said immune response is generated in terms of four-fold seroconversion post vaccination either similar or greater to that of serconversion achieved with the vaccine formulation either in presence of buffers or by administration of antacids.

13. The rotavirus vaccine as claimed in claim 1 and 5, wherein the vaccine further comprises combination of sugars, lactalbumin hydrolysate and recombinant human serum albumin.
14. The rotavirus vaccine as claimed in claim 13, wherein the combination of sugars comprises combination of sucrose, lactose and trehalose.
15. The rotavirus vaccine as claimed in claim 1 and 5, wherein the formulation is a liquid, oral vaccine formulation.
16. A method of generating immune response in a subject against rotavirus comprising orally administering 0.5 ml of an stabilized rotavirus vaccine formulation without any antacid or buffer, the said formulation comprising a rotavirus strain 116E, combination of sugars selected from sucrose, lactose and trehalose, lactalbumin hydrolysate, recombinant human serum albumin, without presence of any buffer formulation in the said rotavirus vaccine formulation.
17. The method of claim 16, wherein the said rotavirus vaccine formulation does not require administration of any antacid or buffer, pre or post administration of the vaccine to the subject.
18. The method of claim 16, wherein the said rotavirus vaccine formulation is capable to withstand the stomach acidity in humans for a period of at least 20 minutes between a range of pH 2 to pH 4.
19. The method of claim 16, wherein the said rotavirus vaccine formulation is stable for a period of at least 60 months at -20°C.
20. The method of claim 16, wherein the said rotavirus vaccine formulation is stable for a period of at least 3 years at 2-8°C, period of atleast 6 months at 25°C, and for a period of at least 4 weeks at 37°C.
21. The method of claim 16, wherein the said vaccine formulation when administered orally to a subject without an antacid or buffer is capable to generate immune response in terms of four-fold seroconversion post vaccination either similar or greater to that of serconversion achieved with the vaccine formulation either in presence of buffers or by administration of antacids.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2017/050237

A. CLASSIFICATION OF SUBJECT MATTER
A61K39/15, A61K39/12 Version=2017.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Patseer, IPO Internal Database, Patentscope, Espacenet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/0068227 A1 (KRISHNA MURTHY ELLA ET AL.) 18 March 2010 (18-03-2010) Paragraphs [0102], [0108]; example 4; claim 1	10, 11
Y	Paragraphs [0102], [0108]; example 4; claim 1 -----	1-9, 12-15
X	WO 2011/007363 A1 (BHARAT BIOTECH INTERNATIONAL LIMITED) 20 January 2011 (20-01-2011) Examples; Claim 1	10, 11
Y	Examples; Claim 1 -----	1-9, 12-15
X	WO 2013/160913 A1 (BHARAT BIOTECH INTERNATIONAL LIMITED) 31 October 2013 (31-10-2013) Whole document	10, 11
Y	Whole document	1-9, 12-15

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search

29-09-2017

Date of mailing of the international search report

29-09-2017

Name and mailing address of the ISA/

Indian Patent Office
Plot No.32, Sector 14, Dwarka, New Delhi-110075
Facsimile No.

Authorized officer

Sunil Kumar

Telephone No. +91-1125300200

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2017/050237

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 16-21
because they relate to subject matter not required to be searched by this Authority, namely:
The subject matter of claims 16-21 relates to a method for treatment of the human body by therapy, which does not require an international search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and Rule 39.1(iv).
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IN2017/050237

Citation	Pub.Date	Family	Pub.Date
US 2010/0068227 A1	18-03-2010	CN 101489587 B	27-05-2015
		US 9642907 B2	09-05-2017
		WO 2007132480 A2	22-11-2007
WO 2011/007363 A1	20-01-2011	CN 102470170 B	18-03-2015
		EP 2453917 B1	22-07-2015
		US 9598676 B2	21-03-2017
WO 2013/160913 A1	31-10-2013	CN 104507495 A	08-04-2015
		EP 2945650 A1	25-11-2015
		US 20150098967 A1	09-04-2015

ADVANCE E-MAIL

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION,
OBTENTION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

HASAN, Afzal
Hasan and Singh
Flat No. 04, Sree Nilayam Apartment
Plot No. 12, Camelot Layout (Near Chirec Public
School),
Kondapur, Hyderabad 500084
INDE

Date of mailing (day/month/year) 25 August 2017 (25.08.2017)	
Applicant's or agent's file reference WO107	IMPORTANT NOTIFICATION
International application No. PCT/IN2017/050237	International filing date (day/month/year) 13 June 2017 (13.06.2017)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 16 June 2016 (16.06.2016)
Applicant BHARAT BIOTECH INTERNATIONAL LIMITED	

The applicant is hereby notified of the date of receipt (or of obtaining by the International Bureau) of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to the date of receipt, **the priority document concerned was submitted or transmitted to or obtained by the International Bureau in compliance with Rule 17.1(a), (b) or (b-bis)**. This Form replaces any previously issued notification concerning submission, transmittal or obtaining of priority documents.

Priority datePriority application No.Country or regional Office
or PCT receiving OfficeDate of receipt
of priority document

16 June 2016 (16.06.2016)

201641020675

IN

15 August 2017 (15.08.2017)

The letters "NR" denote a priority document which, on the date of mailing of this Form, had not yet been received or obtained by the International Bureau in compliance with Rule 17.1(a), (b) or (b-bis). Where the applicant has failed to either submit, request to prepare and transmit, or to request the International Bureau to obtain the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

An asterisk "*" next to a date of receipt, denotes a priority document submitted or transmitted to or obtained by the International Bureau but not in compliance with Rule 17.1(a), (b) or (b-bis) (the priority document was received after the time limit prescribed in Rule 17.1(a); the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b) or the request to the International Bureau to obtain the priority document was made after the applicable time limit under Rule 17.1(b-bis)). Even though the priority document was not furnished in compliance with Rule 17.1(a), (b) or (b-bis), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Kihwan Moon e-mail pct.team1@wipo.int Telephone No. +41 22 338 74 01
Facsimile No. +41 22 338 70 90	

DOCUMENT MADE AVAILABLE UNDER THE PATENT COOPERATION TREATY (PCT)

International application number:	PCT/IN2017/050237
International filing date:	13 June 2017 (13.06.2017)
Document type:	Certified copy of priority document
Document details:	Country/Office: IN
	Number: 201641020675
	Filing date: 16 June 2016 (16.06.2016)
Date of receipt at the International Bureau:	15 August 2017 (15.08.2017)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a),(b) or (b-bis)

PCT/IN2017/050237



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Ph: 011-28032382

Fax: 011-28034301,02

Website: <http://ipindia.nic.in>

E-mail: delhi-patent@nic.in

GOVERNMENT OF INDIA
MINISTRY OF COMMERCE & INDUSTRY
THE PATENT OFFICE
BOUDHIK SAMPADA BHAVAN
PLOT NO.-32, SECTOR-14, DWARKA
NEW DELHI-110078

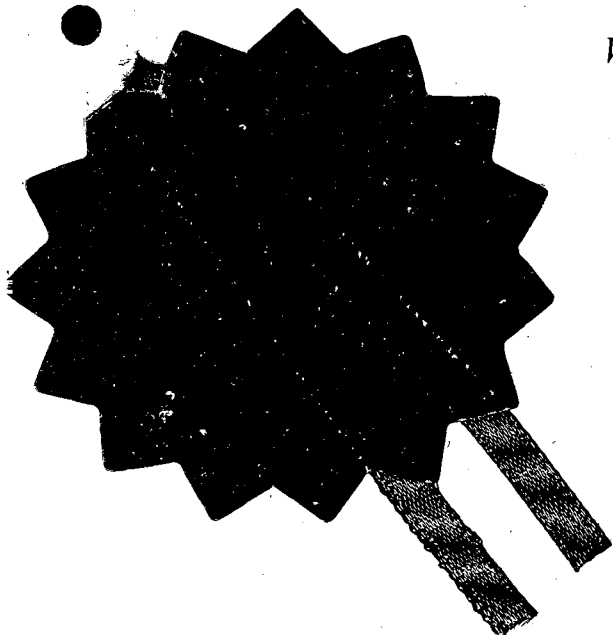
I, the undersigned being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that following documents as annexed hereto are the true copy filed in connection with Indian Patent Application no.201641020675 dated 16th June, 2016.

- (1) Application Form-1*
- (2) Provisional Specification filed on dated 16th June, 2016*
(As per e-filing record)

Witness my hand this 14th day of July, 2017


(Bimi G.B.)

Assistant Controller of Patents & Designs



FORM I THE PATENTS ACT, 1970 (39 of 1970) & THE PATENTS RULES, 2003 APPLICATION FOR GRANT OF PATENT [See sections 7,54 & 135 and rule 20(1)]	(FOR OFFICE USE ONLY) Application No.:..... Filing Date:..... Amount of Fee Paid:..... CBR No.:..... Signature:.....
--	--

1. APPLICANT(S)

Sr.	Name	Nationality	Address
1	Bharat Biotech International Limited	India	Genome Valley, Turkapally, Shameerpet, Hyderabad- 500 078, India.

2. INVENTOR(S)

Sr.	Name	Nationality	Address
1	Krishna Mohan VADREU	India	Bharat Biotech International Limited, Genome Valley, Turkapally, Shameerpet, Hyderabad- 500 078, India.
2	Sai Devarajulu PRASAD	India	Bharat Biotech International Limited, Genome Valley, Turkapally, Shameerpet, Hyderabad- 500 078, India.
3	Krishna Murthy ELLA	India	Bharat Biotech International Limited, Genome Valley, Turkapally, Shameerpet, Hyderabad- 500 078, India.

3. TITLE OF THE INVENTION: A BUFFER FREE, ACID STABLE, LOW DOSE VOLUME ROTAVIRUS VACCINE**4. ADDRESS FOR CORRESPONDENCE OF APPLICANT / AUTHORISED PATENT AGENT IN INDIA**

HASAN AND SINGH, Flat No. 04, Sree Nilayam Apartment, Plot No. 12, Camelot Layout (Near Chirec Public School), Kondapur, Hyderabad- 500084, India Phone: +91-40-65189786, 23019786 / Cell: +91-9492033581 Fax: +91-40-23013786 E Mail: afzal@hasanandsingh.com / hasan@hasanandsingh.com

Telephone No.: +91-9492033581

Fax No.: +91-40-23013786

Mobile No.:

E-mail: hasan@hasanandsingh.com

5. PRIORITY PARTICULARS OF THE APPLICATION(S) FILED IN CONVENTION COUNTRY:

Sr.	Country	Application Number	Filing Date	Name of the Applicant	Title of the Invention
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6. PARTICULARS FOR FILING PATENT COOPERATION TREATY (PCT) NATIONAL PHASE APPLICATION

International Application Number	International Filing Date as Allotted by the Receiving Office
PCT//	

7. PARTICULARS FOR FILING DIVISIONAL APPLICATION

Original (first) Application Number	Date of Filing of Original (first) Application
-------------------------------------	--

8. PARTICULARS FOR FILING PATENT OF ADDITION

Main Application / Patent Number	Date of Filing of Main Application
----------------------------------	------------------------------------

9. DECLARATIONS:

(i) Declaration by the inventor(s)

I/We ,Krishna Mohan VADREVU,Sai Devarajulu PRASAD,Krishna Murthy ELLA, is/are the true & first inventor(s) for this invention and declare that the applicant(s) herein is/are my/our assignee or legal representative.

(a) Date:16/06/2016

(b) Signature(s) of the inventor(s):.....

(c) Name(s):Krishna Mohan VADREVU,Sai Devarajulu PRASAD,Krishna Murthy ELLA

(ii) Declaration by the applicant(s) in the convention country

I/We, the applicant(s) in the convention country declare that the applicant(s) herein is/are my/our assignee or legal representative.

(a) Date:16/06/2016

(b) Signature(s) :.....

(c) Name(s) of the singnatory:Bharat Biotech International Limited

(iii) Declaration by the applicant(s):

- The Provisional specification relating to the invention is filed with this application.
- I am/We are, in the possession of the above mentioned invention.
- There is no lawful ground of objection to the grant of the Patent to me/us.
- I am/We are, the assignee or legal representative to true first inventors.

10. FOLLOWING ARE THE ATTACHMENTS WITH THE APPLICATION:

Sr.	Document Description	FileName
1	PROVISIONAL SPECIFICATION	PROVISIONAL SPECIFICATION.pdf
2	STATEMENT OF UNDERTAKING (FORM 3)	FORM-3.pdf
3	DECLARATION OF INVENTORSHIP (FORM 5)	FORM-5.pdf

I/We hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated hering are correct and I/We request that a patent may be granted to me/us for the said invention.

Dated this 16/06/2016 day of 2016

Signature:.....

Name:AFZAL HASAN

To The Controller of Patents
The Patent office at CHE

This form is digitally signed.

FORM 2

**THE PATENTS ACT, 1970
(39 of 1970)**

&

THE PATENTS RULES, 2006

PROVISIONAL SPECIFICATION

(See Section 10 and rule 13)

**“A BUFFER FREE, ACID STABLE, LOW DOSE VOLUME
ROTAVIRUS VACCINE”**

APPLICANT : Bharat Biotech International Limited
Nationality : India
**Address : Genome Valley, Turkapally, Shameerpet,
Hyderabad - 500078, India.**

The following specification describes the invention:

FIELD OF THE INVENTION

The invention relates to the field of rotavirus vaccines. More particularly, this invention relates to the field of rotavirus vaccines which are buffer-free or do not require any buffer pre, during or post administration of the rotavirus vaccine. This invention also relates to the field of amount of dose volume per unit vaccine, particularly rotavirus vaccines.

BACKGROUND OF THE INVENTION

It is well known that Rotaviruses are the leading cause of severe Gastroenteritis in infants in children world wide. Even improvements in hygiene, water quality and sanitation that generally control many bacteriae and parasites which are responsible for causing other types of diarrhea do not adequately prevent the spread of rotavirus. Therefore, vaccination is the only strategy capable of significantly reducing the disease burden caused by rotavirus infections in underdeveloped and developing countries across the globe. Vaccination is particularly significant for the countries which lack the required economic support and infrastructure to address the rotavirus infections in the infected patients. Till date, there have been at least two commercialized rotavirus vaccines in the world, the first among those being Rotashield® supplied by Wyeth Holdings, in the year 1999. This was a live, oral, tetravalent rotavirus vaccine produced using 1 rhesus monkey rotavirus, 3 rhesus-human reassortant rotavirus vaccine licensed in the United States. However, it was withdrawn from the market after a few months because of its association with adverse affects of intussusception.

The Rotashield® comprised a composition containing, sucrose, monosodium glutamate, potassium monophosphate, potassium diphosphate, fetal bovine serum and neomycin sulphate in amphotericin medium. Corresponding patent publication on rotavirus vaccine compositions WO2000006196A2, teaches that the vaccine compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sodium monolaurate, tri-ethanolamine oleate, citrate- bicarbonate, or the like. When the composition is administered orally it may also be necessary to provide the individual a buffer solution to partially neutralize stomach acid and protect the reassortant rotavirus while passing through the intestine. Buffer solutions appropriate for this use include sodium bicarbonate, calcium bicarbonate, or the like

RotaTeq®, is a pentavalent human-bovine (WC3) reassortant live-attenuated, oral vaccine manufactured by Merck & Co., comprising a Bovine rotavirus strain WC3, P7[5]G6 involving 5 reassortants; out of which 4 reassortants with the VP7 gene from G1, G2, G3, or G4 and 1 reassortant with the VP4 P1A[8] gene from the human rotavirus parent strain with the remainder of the genes from the WC3 bovine rotavirus parent. The vaccine formulation (corresponding patent publications of Merck viz. WO1998/13065, WO2001/08495 and WO2002/011540) contains certain stabilizers in a tissue culture media along with the presence of buffers in the formulation itself, the said buffer being sodium citrate and sodium phosphate. The carboxylate of the patented claims are being used and acts as a buffer to make the rotavirus antigen withstand the stomach acidity while the virus antigen gets administered to the infant's stomach.

Further, Rotarix® is a monovalent vaccine, comprising a passaged Human rotavirus strain RIX4414 derived from the 89-12 strain. Rotarix® (produced by GlaxoSmithKline Biologicals), is a live vaccine that contains the attenuated monovalent G1, P[8] human rotavirus strain. The vaccine is available in lyophilized form mixed with stabilizers, to be reconstituted with a calcium carbonate based buffer diluent prior to vaccine administration into the subjects.

Rotarix was prepared by reconstituting the lyophilized active ingredient (RIX4414 strain + excipients) with 1.0 ml of GSK's CaCO₃ buffer contained in a pre-filled syringe. After injecting the buffer into the vial containing the lyophilized vaccine, the vial was shaken well to resuspend the vaccine. The entire volume of resuspended product was then withdrawn into the same syringe, the needle (or transfer device) of the syringe was discarded, and the product was administered promptly as a single oral dose. Alternatively, reconstitution of the commercial lyophilized vaccine will follow the same procedures, using an oral applicator filled with buffer and a transfer adapter instead of a syringe and needle. The reconstituted vaccine should appear milky white in appearance. It should be inspected visually for particulate matter and/or discoloration, and not be administered if either of these conditions exists. Rotarix is to be administered orally. The infant should be seated in a reclining position, and the entire content of the oral applicator containing the reconstituted vaccine should be administered on the inside of the cheek. Rotarix is not to be administered by injection. Hence, it is apparent that, reconstitution of buffer diluent contains so many steps, right from ensuring that the reconstitution has been properly done to the point of administration of the

vaccine. All these factors definitely, cause decrease in vaccine compliance, amounting to partial protection from rotavirus infection rather than achieving a complete protection.

Corresponding patent applications from GSK on rotavirus vaccine formulations WO2001/012797, WO2005/02133, WO2006/087205, which involves a live attenuated rotavirus strain P43 serially passaged with specific mutations also supplemented with an antacid buffer for reconstitution. The said rotavirus strain G protein (VP4 and VP7) capable to induce immune response to atleast 4 other rotavirus strains to G1 and atleast one of G14 serotypes.

Other patented rotavirus vaccine compositions with stabilizers and buffers by Bharat Biotech International Limited with an Indian asymptomatic strain 116E isolated from a human child have also been disclosed. These require separate administration of an antacid buffer to vaccine recipient prior to receiving the vaccine to increase the pH of the stomach, wherein said antacid buffer is a citrate-phosphate buffer, in order to neutralize the stomach acidity at the time of administration of the rotavirus vaccine antigen. Patent Publication WO2013160913, provides rotavirus vaccine compositions comprising rotavirus antigen, stabilizers and certain novel buffer combinations. The buffers in the invention are pre-mixed in the rotavirus vaccine compositions to neutralize the high acidic pH of the stomach without requiring separate administration of an antacid before vaccine administration.

Thus it is evident that different rotavirus vaccines have been developed using different strains, both human and animal reassortant types. There are a few important differences in characteristics of these strains, typical ones, as listed below.

Rotavirus vaccines with human strains	Animal reassortant rotavirus vaccines
Natural infection offers protection against subsequent rotavirus disease.	Animal rotaviruses are naturally attenuated strains, which lowers replication in humans.
Human viruses have high replication capacity in the host, and therefore attenuated by multiple tissue culture passage in multiple hosts. Since, these are human strains, a vaccine titer of 10^4 FFU/0.5 ml is enough to induce	Rotavirus vaccines with animal reassortant strains require very high vaccine titers to be able to induce the required immune response

required immunogenecity against rotavirus infections in humans.	
Broad immunity is acquired through various immune effector mechanisms.	Expectation that neutralizing antibody in the gut lumen is required. Immunity obtained is not high as vaccines with human strains.
Heterotypic protection is gained through broad immune response. This means, it offers cross protection across different strains.	Reassortant vaccine constructs to include the common human rotavirus antigens.

Table 1: Differences between Human Monovalent and Animal reassortant vaccines

RATIONALE BEHIND THE PRESENT INVENTION

Thus it is known that rotavirus is highly sensitive to acidic environments [Estes, M. Graham, D., Smith, E. and Gerba, C. (1979). Rotavirus Stability and Inactivation. *Journal of General Virology*, 43(2), pp.403-409). It has also been reported that infantile gastroenteritis virus becomes unstable and there is a collapse of their outer shell and capsomeres when subjected to environment having pH less than 3.0 [Palmer, E., Martin, M. and Murphy, F. (1977). Morphology and Stability of Infantile Gastroenteritis Virus: Comparison with Reovirus and Bluetongue Virus. *Journal of General Virology*, 35(3), pp.403-414). It has also been reported that many strains of rotavirus have been inactivated within a minute when exposed to environment having pH 2, and similar results were obtained with human gastric juice having pH 1.8 (Weiss, C. and Clark, H. (1985). Rapid Inactivation of Rotaviruses by Exposure to Acid Buffer or Acidic Gastric Juice. *Journal of General Virology*, 66(12), pp.2725-2730). It has further been reported that human serotype 1 rotavirus is completely inactivated when subjected to an environment of pH 2.5 (Meng, Z., Birch, C., Heath, R. and Gust, I. (1987). Physicochemical Stability and Inactivation of Human and Simian Rotaviruses. *Applied and Environmental Microbiology*, 53(4), pp.727-730). Rotavirus, in general, is known to be highly unstable when subjected to acidic environments and rapidly gets inactivated. Inactivation rates of three bovine and several primate-origin rotaviruses were determined during exposure to acid buffers at pH 2.0, pH 3.0 or pH 4.0. Each rotavirus was inactivated at pH 2.0 (the acidity most resembling the normal fasting stomach) very rapidly

with half-lives for infectivity determined to be 1-min or less. Each rotavirus was inactivated at a much slower rate at pH 3.0; inactivation at pH 4.0 was minimal. Some differences in acid resistance between different rotavirus strains were detected. Although these determinations were performed at room temperature (23°C), experiments at diverse temperatures indicated an even more rapid rate of viral inactivation by acid at normal body temperature (37°C). Studies of rotavirus exposed to natural human gastric juice at pH 1.8 or pH 2.1, revealed a rate of virus inactivation similar to that observed with glycine buffer of identical pH (Ref: Geigy Scientific Tables, volume 1, 1981, page 126).

Rotavirus inactivation takes place between pH 2.0 to pH 3.0 but at pH 4.0 no or minimal inactivation takes place. Since, the stomach contains highly gastric acid environment, it has been imperative to add buffers into the rotavirus vaccine formulations till date. Buffers in rotavirus formulations aid in retaining a constant pH of the rotavirus vaccine formulation, thus enabling antacid or buffering capacity of the vaccine formulations. The antacid capacity (also termed as acid neutralization capacity) of the rotavirus vaccines have been measured by Baby Rossett Rice Assays in the GSK's patents. The acid neutralization capacity of a given formulation is defined as the time measured to maintain the pH above 4.0 and is evaluated by Baby Rossette Rice assay. Baby Rosset assay reportedly is alleged to simulate the gastric environment of the human stomach. The latest patent by GSK US8821891B2 advocates a lower concentration of phosphates, and with an increased concentration of a carboxylate, preferably adipate ranging from 100 mM to 1M, preferably 400 mM to 700 mM in a dose volume as low as 1.5 ml per human dose of rotavirus vaccine. Adipate acts as a buffer capable to withstand the high stomach acidity (pH 1 to 2). The capacity to withstand human stomach acidity is measured by Baby Rosset Rice Assay, having a value of 8 to 23 minutes which is said to be capable and good enough to avoid getting inactivation of the rotavirus antigen in the vaccine formulation after administration of the rotavirus vaccine into a human infant.

Thus, to effectively, administer a rotavirus vaccine which is capable to elicit immunogenic response sufficient enough to confer protection against rotavirus infections, at least 1.5 to 2 ml of the rotavirus vaccine or reconstituted vaccine formulation per human dose for oral administration is needed to effectively allow the rotavirus antigen to pass through the highly acidic environment of the stomach. Therefore, by the above descriptions of the status of the present nature of the rotavirus vaccines available till date, it would also be easily understood

and acknowledged by the persons having skilled in the art, that rotavirus antigen is a highly acid labile antigen, and administration of rotavirus vaccine always requires an antacid buffer component thereby increasing the required quantity (in terms of dosage in volumes per unit vaccine) of vaccine administration.

It is also a fact that the rotavirus antigen is a highly unstable virus and considering the situation that live attenuated rotavirus vaccine antigen would be more unstable; therefore, rotavirus antigen are being supplemented with respective proprietary vaccine stabilizers to stabilize the vaccine formulations, which add up to the amount of the vaccine dose in terms of volume per unit vaccine. Furthermore, inclusion of buffers or antacids or requirement of reconstitution of the vaccine with a buffer based vaccine diluent increases the amount of vaccine dose in terms of volume per unit vaccine. Hence, providing a rotavirus vaccine without a buffer/antacid will definitely reduce the dosage volume per unit vaccine. At the same time, it is imperative that such a rotavirus vaccine without a buffer should not be compromising with the efficacy as well as stability of rotavirus vaccine.

Since, the subject of the rotavirus vaccine recipient is always an infant within the age group between 6 weeks to 6 months of age, there is every possibility that a considerable amount of rotavirus vaccine formulation delivered into the mouth of the infant gets spilled out. This creates a chance of partial administration of the vaccine thereby reducing vaccine compliance and not protecting the disease burden in spite of receiving the vaccine. Therefore, it is always desirable that the amount of dosage in terms of volume per unit rotavirus vaccine is reduced as much as possible without compromising the efficacy of the vaccine formulation. Currently, the least dosage volume disclosed in the state of the art for rotavirus vaccine range from 1ml-2.5 ml. The vaccines contain vaccine stabilizers and vaccine buffers adding up to the vaccine dose volume. So, decrease in dosage volume per unit rotavirus vaccine having the same capacity to raise required immunogenicity for prophylaxis against rotavirus infection in infants will decrease the possibility of spilling out of the vaccine formulation administered to the infant. It would therefore be highly non-obvious to make available a low dose rotavirus vaccine formulation up to 1ml, preferably only 0.8ml, or most preferably 0.5 ml only as a human dose without a buffer which is equally or rather, more effective than the rotavirus vaccine(s) made available with buffers or antacids having high dosage volume per unit vaccine.

The prior art discloses all rotavirus vaccine formulations all of which are to be stabilized in liquid formulations at refrigerated temperatures between $5\pm 3^{\circ}\text{C}$ or as lyophilized powders which require reconstitution of the vaccine prior to the administration of the vaccine with a suitable diluent. It has been already discussed that, reconstitution of the vaccine from lyophilized powder requires specific skill, and care. Further, separate manufacturing requirements for diluents along with the vaccine doubles the vaccine manufacturing costs as well as transportation costs. Deficiency in skill, care and appropriate infrastructure and warehouse requirements adds up to the problem with lyophilized rotavirus vaccine supply. For liquid vaccines at $5\pm 3^{\circ}\text{C}$, the shelf life of the vaccines are limited to only up to 2 years. There is also an associated titer loss with vaccines made available at $2-8^{\circ}\text{C}$, during storage and transportation which is undesirable. But, rotavirus vaccine formulations at -20°C , it has been found that there has been no transportation and storage loss at all. The chances of vaccine contamination is also reduced to much greater extent for vaccines at -20°C . Further, the vaccine supply facility at -20°C has been already established in almost 120 countries across the World for polio vaccine supplies. Therefore, according to existing facilities for vaccine storage and vaccine supplies similar to polio vaccine supplies would greatly help in reducing the rotavirus disease burden in the World. It is desirable that alternative strategies be developed wherein the rotavirus vaccine stability be further increased, so that the vaccine may be made available to remotest corners of the globe. Therefore, novel rotavirus vaccine formulations are being disclosed in this patent application, wherein the rotavirus vaccine formulations are stable for at least 5 years, being absolutely new and not stated in the current state of the art at all.

OBJECTS OF THE INVENTION

The primary objective of the invention is to provide a buffer free and acid stable rotavirus vaccine.

One more primary objective of the invention is to provide a low dose volume up to 1 ml or even only 0.8 ml, preferably a 0.5ml low dose volume rotavirus vaccine.

Another objective of the invention is to provide a stable rotavirus vaccine composition with a vaccine volume of as low as 0.5 ml per dose.

Another objective of the invention is to reduce spilling out of rotavirus vaccine a

minimizing associated vaccine wastage by the vaccine recipient and increase vaccine compliance thereby.

Another objective of the invention is to make a rotavirus vaccine composition that does not require any specific dilution or reconstitution techniques prior to or during vaccine administration.

One more objective of the invention is to provide a rotavirus vaccine which is stable for at least 5 years at -20°C .

A further objective of the invention is provide a low dose volume rotavirus vaccine which is stable for 2 years at $5\pm 3^{\circ}\text{C}$, and 6 months at 25°C , and 1 week at 37°C .

One further objective of the invention is to provide a buffer free, low dose volume upto 1ml rotavirus vaccine composition which is stable in stomach at pH 2 to pH 4 for atleast 20 minutes.

Another objective of the invention is to provide a buffer free, low dose volume up to 1 ml rotavirus vaccine composition which is stable at acidic pH of 2-4 without any supplement of buffers or acid stabilizing agents.

A further objective of the invention is to provide a buffer free, acid stable, low dose volume rotavirus vaccine composition which does not require administration of any antacid or buffer, pre or post or during administration of the vaccine to the subject.

Alternatively, it is also an objective of the invention is to provide a low dose volume rotavirus vaccine of 0.5 ml along with the presence of a buffer, which is equally effective against rotavirus infections in humans.

Further objective of the invention is to provide a low dose volume rotavirus vaccine without a buffer, which is capable to generate either equivalent or greater immune response than rotavirus vaccines which are supplemented with antacids or buffers in vaccine compositions.

SUMMARY OF THE INVENTION

According to one embodiment of the invention provides a vaccine composition wherein the

loss in rotavirus vaccine titer is not more than 0.55 FFU/ per 0.5 ml between pH of 2-4 for a period of at least one hour.

In a further embodiment, the invention provides a vaccine composition wherein the loss in rotavirus vaccine titer is not more than 0.68 FFU/ per 0.5 ml between pH of 2-4 for a period of at least 20 minutes.

One more embodiment of the invention, provides various rotavirus vaccine composition and dose volume ranging from 1 ml to 0.5 ml, wherein the vaccine formulations are without buffers or antacids, and exemplifies nominal drop in vaccine titer at pH 2 and pH 4 for a time span of 30 minutes.

One another embodiment of the invention, provides acid neutralization capacities of a 1 ml dose volume rotavirus composition between 0.5 ml to 1 ml per unit vaccine in comparison with rotavirus vaccine compositions in presence of buffers established through Baby Rosset R₅₀ assays.

A further embodiment of the invention, provides a buffer free rotavirus vaccine of dose volume only 0.5 ml which is capable to generate either equivalent or greater immune response in terms of four-fold seroconversion post vaccination similar to that achieved with the vaccine formulation either in presence of buffers or without administration of antacids.

In another embodiment, the invention provides a method of prophylaxis against rotavirus diarrhea in humans, the method comprising steps of administering an effective amount of a stabilized rotavirus vaccine formulation comprising rotavirus strain 116E, combination of sugars selected from sucrose, lactose and trehalose, lactalbumin hydrolysate, recombinant human serum albumin, without the presence of any buffer formulation in the said rotavirus vaccine formulation.

One more embodiment of the invention provides for stable rotavirus vaccine compositions which are stable at -20°C for at least 5 years.

A further embodiment of the invention provides stable rotavirus vaccine compositions at

8°C, 25 °C and 37°C for 2 years; 6 months and 3 weeks respectively.

DETAILED DESCRIPTION OF THE INVENTION

Embodiment 1: Buffered Rotavirus Formulations and Gastric acidity

It has been mentioned above in the background of the invention that, usage of buffers is a common practice rotavirus vaccine formulation strategies, that has being undertaken by various rotavirus vaccine manufacturers till date, and hence the Rota vaccine comprising the 116E strain at -20°C formulation (SPG and DMEM) has been tested with citrate-bicarbonate buffer, and normal saline conditions initially.

Bharat Biotech International Limited obtained the human rotavirus strains 116E from National Institute of Health under a Material Transfer Agreement with National Institute of Allergy and Infectious Diseases (NIAID), NIH, Bethesda, USA. The complete genomic sequence of rotavirus strains 116E is already reported in literature.

Three Rotavirus vaccine formulations with rotavirus 116E stored at -20°C were taken in 3 groups (Group I, II and III) as detailed below. The rotavirus vaccine compositions in all the three groups below comprise of live attenuated Rotavirus antigen of the strain 116E dissolved in 10% SPG (sucrose, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate) and Dulbecco's Minimum Essential Medium. The said vaccine formulations were treated separately as detailed below:

Group I : 0.5 ml of Rotavirus 116 E formulation A at -20°C.

Group II : 2 ml (equivalent 4 human doses of 0.5ml per human dose) of Rotavirus 116 E formulation A mixed with 5 ml of Normal Saline.

Group III : 0.570 ml of Rotavirus 116 E formulation A + mix of [1.42ml Citrate -Bicarbonate buffer + 8 ml of 34.8 mEq HCl].

As given above, Group I includes the rotavirus vaccine formulation at -20°C wherein no buffer or saline was added to the vaccine formulation and the vaccine titer was measured for

next 120 minutes. IN Group II, 2 ml of rotavirus 116E vaccine formulation (stored at -20°C) was taken and added with 5 ml of normal saline, and vaccine titer was measured at a target titer of 10^6 FFU/ml upto 120 minutes. In Group III, 0.57 ml of rotavirus 116E vaccine formulation (stored at -20°C) was taken and added with 1.42 ml of Citrate Bicarbonate Buffer and 8 ml of 34.8 mEq Hydrochloric acid.

Group#	0 mins in FFU/0.5 ml	60mins in FFU/ml	120mins in FFU/ml	Remarks
Group I	$10^{6.35}$	$10^{6.17}$	$10^{6.32}$	Highly stable
Group II	$10^{5.76}$	$10^{5.84}$	$10^{5.69}$	Fairly stable
Group III	$10^{4.61}$	$10^{5.30}$	$10^{5.11}$	Fairly stable

Table 2: Rotavirus vaccine formulation (at storage temperature -20°C) vaccine titers in presence of buffers at 0 minute, 60 minute and 120 minute.

Therefore, the above table establishes that both for exposing the virus to normal saline conditions or in presence of buffer solutions, the rotavirus antigen titer is stable, and therefore the vaccine is stable.

Embodiment 2: Immunoperoxidase Assay of Rotavirus Vaccine Formulations at different pHs.

Example 1: Immunoperoxidase Assay was done after subjecting the rotavirus vaccine formulations directly to the acidic environment by direct addition of the 34.8 mEq of Hydrochloric Acid (HCl) to the rotavirus vaccine formulation. The pH of the vaccine formulations were directly brought down from pH 7 to various lower pH values of pH 2, and pH 4 by direct addition of acid, and subsequently the vaccine antigen titer was measured at the given lower pH values of pH 2, and pH 4. Following this, the vaccine titer was measured for upto 1 hour at various lower pH of 2 and 4. Following observations were made. The reaction mixture was studied at various time points and various pH levels. Details are shown below in the table below. The said rotavirus vaccine formulations (at storage temperature -20°C) contains live attenuated rotavirus antigen 116E, 10% SPG (sodium phosphate

glutamate) dissolved in Dulbecco's Minimum Essential Medium.

Sample details	Titer in FFU/0.5 mL dose					R
	Actual titer at 0 minutes	Corrected Titer with Dilution Factor at 0 minutes	Actual titer after 1 hour	Corrected Titer with Dilution Factor after 1 hour	Log loss after 1 hour.	
0.5 ml Rotavirus 116E vaccine formulation at pH 7.4	$10^{4.95}$	NA	$10^{4.94}$	$10^{4.94}$	-	-
0.5 ml Rotavirus 116E vaccine formulation + 34.8 mEq HCl at pH 4.0	$10^{3.58}$	$10^{5.05}$	$10^{3.58}$	$10^{5.05}$	Nil	N of
0.5 ml Rotavirus 116E vaccine formulation + 34.8 mEq HCl at pH 2.0	$10^{3.54}$	$10^{5.01}$	$10^{2.93}$	$10^{4.40}$	0.55	M ef ph

Table 3: Immunoperoxidase Assay results at a target titer of $10^{5.0}$ FFU/0.5 ml dose at 0 minutes and 60 minutes by direct addition of acid to rotavirus vaccine formulations (storage temperature -20°C).

Note: Calculated reduction in Infectivity Titer value due to dilution = $10^{1.47}$ FFU. Therefore, actual vaccine titer after dilution is $10^{2.93}$ at pH 2. All the samples were titrated in triplicate by Immunoperoxidase Assay. The oral rotavirus vaccine (target titer $10^{5.0}$ FFU / 0.5 mL) was stable at pH 4.0. Target Titer means, the minimum concentration of the rotavirus 116E antigen in the vaccine vial intended to be attained in the given vial. The original titer at neutral pH of the vaccine is $10^{4.95}$ FFU/0.5 ml at 0 minutes. The vaccine titer at pH 2 at 0 minutes is $10^{3.54}$ FFU/0.5 ml and the titer dropped to $10^{2.93}$ FFU/0.5 ml within 1 hour. After adding the dilution factor value of 1.47 due to addition of the acid, with the actual titer value ($10^{2.93}$ FFU/0.5ml), the corrected vaccine titer at pH 2 after 1 hour from addition of acid is $10^{4.40}$ as compared to the original vaccine titer of $10^{4.95}$ at pH 7 at 0 minutes. Therefore, the vaccine experienced a total log loss in vaccine titer of 0.55 only at pH 2 within a period of 1 hour. This establishes that, even at lower pH value of 2, that is to say

specific acidic environment as it appears in the human stomach, the rotavirus vaccine formulation is capable to sustain its vaccine titer at a value of $10^{4.40}$ FFU/0.5 ml. At this vaccine titer, the vaccine is capable to raise sufficient immune response against subsequent rotavirus infections.

Example 2: The same experiment has been repeated with a different vaccine (at storage temperature -20°C), wherein the target titer is $10^{6.0}$ FFU/ 0.5 ml (dose) Calculated values along with dilution factor are provided in the following table at various pH values at 0 minutes and after 1 hour. The said rotavirus vaccine formulations (at storage temperature 20°C) contains live attenuated rotavirus antigen 116E, 10% SPG dissolved in DMEM.

Vaccine Sample Details	pH	'Corrected Titer with Dilution Factor at 0' Hr in FFU/0.5 ml	Corrected Titer with Dilution Factor after 1 hour in FFU/0.5ml	Log loss after 1 hour
0.5 ml Rotavirus 116E vaccine formulation	7.2	$10^{5.35}$	$10^{5.30}$	0.05 (no loss)
0.5 ml Rotavirus 116E vaccine formulation + 34.8 mEq HCl	4.0	$10^{5.33}$	$10^{5.09}$	0.24 (marginal loss)
0.5 ml Rotavirus 116E vaccine formulation + 34.8 mEq HCl	2.0	$10^{4.89}$	$10^{4.21}$	0.68 (marginal loss)

Table 4 : Immunoperoxidase Assay results at a target titer of $10^{6.0}$ FFU/0.5 ml dose at 0 minutes and 60 minutes by direct addition of acid to rotavirus vaccine formulations at storage temperature -20°C).

Conclusion: There was only loss in 0.24 log loss in vaccine titer in vaccine samples at pH 4.0 which is considered to be no effect on pH in practical circumstances kept for 1 hour. At pH 2.0, there was a 0.46 Log titer loss in the titer at 0 hour and a loss of 0.68 log₁₀ FFU/0.5 ml after 1 hour. This is said to be having very marginal effect on pH on the vaccine titer values at storage temperature -20°C at an acidic lower pH value of 2.

According to the above table, we see that vaccine titer of $10^{4.89}$ FFU/0.5ml at pH 2.0 is retained till a titer value of $10^{4.21}$ FFU/0.5 ml, a log titer of 4.89-4.21 is good enough.

replication of the rotavirus in the gut of an infant stomach at an age of 6 weeks to 2 years. The stomach acidity of an infant is also pH 2, representing a simulated gastric environment. The vaccine is still able to survive having a titer of 4.89 Log 10 FFU/0.5 ml, which is enough to cross the stomach and then get established into the intestinal villi and for further replication of rotavirus, responsible for generating immune response in the infant, thereby conferring protection and prevention of rotaviral gastroenteritis. Therefore, the vaccine was found to be stable at acidic pH of 2 for at least 1 hour duration without having any considerable loss in vaccine titer at all.

Example 3: In vitro Experiment to demonstrate that Rotavirus 116E vaccine does not require antacid : Apart from the conclusions from the Clinical trial results that follow in the patent specification, it was also proved in vitro simulating similar conditions of stomach acidity administering the vaccine directly without buffer.

Example 3.1: 1 mL of vaccine (formulation 3A) containing rotavirus 116E, combination of sucrose (50%), lactose (0.5%) and trehalose (0.5%), lactalbumin hydrolysate (1 %), human serum albumin (0.4%) , was taken and added with 1.25 mL of 0.1 N HCl and mixed well. Sample was taken and tested for virus content at various pH levels at different time intervals. The results are shown in the table 5.1 below.

S.No	Sample details	Virus content (FFU/mL)
1	pH 2.0-0 minutes	5.72
2	pH 2.0- 10 minutes	4.78
3	pH 2.0- 30 minutes	3.84
4	pH 3.0-0 minutes	5.96
5	pH 3.0- 10 minutes	5.96
6	pH 3.0- 30 minutes	5.91
7	Control	6.10

Table 5 1 Immunoperoxidase assay of 1ml vaccine with rotavirus 116E (formulation 3A)

Example 3.2: 0.8 mL of vaccine containing rotavirus 116E, combination of sucrose (40%) and trehalose (0.5%), lactalbumin hydrolysate (20%), polyvinyl pyrrolidone (0.5%) (formulation 3B) was taken and added with 1.25 mL of 0.1 N HCl and mixed well. Sample was taken and tested for virus content at various pH levels at different time intervals. The results are shown in the table 5.2 below.

S.No	Sample details	Virus content (FFU/mL)	Virus content (FFU/0.8mL)
1	pH 2.0-0 minutes	4.71	4.62
2	pH 2.0- 10 minutes	4.69	4.60
3	pH 2.0- 30 minutes	4.41	4.32
4	pH 3.0-0 minutes	5.97	5.88
5	pH 3.0- 10 minutes	5.94	5.85
6	pH 3.0- 30 minutes	5.93	5.84
7	Control	6.10	

Table 5.2: Immunoperoxidase assay of 0.8 ml vaccine with rotavirus 116E (formulation 3B)

Example 3.3: 0.5 mL of Rotavirus 116E vaccine containing combination of sucrose (60%) and trehalose (0.5%), soy protein (20%), pyridoxine-HCL (0.1%), polyvinyl pyrrolidone (0.25%) (formulation 3C) was taken and added with 1.25 mL of 0.1 N HCl and mixed well. Sample was taken and tested for virus content at various pH levels at different time intervals. The results are shown in the table 5.3 below.

S.No	Sample details	Virus content (FFU/0.5 mL)
1	pH 2.0-0 minutes	4.29
2	pH 2.0- 10 minutes	4.21
3	pH 2.0- 30 minutes	3.39

4	pH 3.0-0 minutes	5.20
5	pH 3.0- 10 minutes	5.17
6	pH 3.0- 30 minutes	5.08
7	Control	5.80

Table 5.3: Immunoperoxidase assay of 0.8 ml vaccine with rotavirus 116E (formulation 3C)

Example 3.4: 0.5 mL of Rotavirus 116E vaccine containing 10% SPG dissolved in DME (formulation 3D) was taken and added with 1.25 mL of 0.1 N HCl and mixed well. Sample was taken and tested for virus content at various pH levels at different time intervals. The results are shown in the table 5.4 below.

S.No	Sample details	Virus content (FFU/0.5 mL)
1	pH 2.0-0 minutes	4.78
2	pH 2.0- 10 minutes	4.48
3	pH 2.0- 30 minutes	4.29
4	pH 3.0-0 minutes	5.22
5	pH 3.0- 10 minutes	5.17
6	pH 3.0- 30 minutes	4.70
7	Control	5.80

Table 5.4: Immunoperoxidase assay of 0.8 ml vaccine with rotavirus 116E (formulation 3D)

Example 3.5: 0.5 mL of Rotavirus 116E vaccine containing sucrose (40%), trehalose (0.5%), lactose (5%), rHSA (0.5%), LAH (1%) and mixed buffer (ammonium acetate + ammonium bicarbonate + diammonium orthophosphate) (formulation 3D) was taken and added with 1.25 mL of 0.1 N HCl and mixed well. Sample was taken and tested for virus content at various pH levels at different time intervals. The results are shown in the table 5.5 below

S.No	Sample details	Virus content (FFU/0.5 mL)
1	pH 2.0-0 minutes	4.77
2	pH 2.0- 10 minutes	4.65
3	pH 2.0- 30 minutes	4.39
4	pH 3.0-0 minutes	5.21
5	pH 3.0- 10 minutes	5.19
6	pH 3.0- 30 minutes	4.87
7	Control	5.80

Table 5.5: Immunoperoxidase assay of 0.8 ml vaccine with rotavirus 116E (formulation 3D)

Conclusions: From the above tables it is evident that at pH 2.0 for 30 minutes also a Rotavirus 116E vaccine formulations are able to withstand in absence of any buffer or antacid.

Embodiment 4: Baby Rossett Rice Analysis

BRR assay is the validated procedure for acid neutralization capacity of rotavirus vaccine formulations. So selected vaccine formulations containing Citrate Phosphate Buffer and Citrate Bicarbonate Buffer are evaluated for Acid Neutalization Capacity using this assay. In vitro experiments were conducted under simulated conditions of infantile stomach using Baby Rosette Rice Assay (BRR). Various formulations with various combinations of buffering agents were selected for the acid neutralization experiments and the results are given in the table below at various pH values wherein the conditions were simulated as infantile stomach. The vaccine formulations were diluted with water for injection upto 10 ml, then added 4 ml of 0.1 N HCl, then 0.5 ml of 0.1 N HCl added per minute until pH reached 4.0. ANC is defined as the time in minutes taken to maintain the pH above 4.0.

Vaccine Formulation 4A	Rotavirus antigen 116E target titer of 10^6 FFU/0.5ml; 1 ml of DMEM (quantity sufficient)
Vaccine Formulation 4B	Rotavirus antigen 116E target titer of 10^6 FFU/0.5ml; 1 ml of DMEM (quantity sufficient) added to 2.5 ml of Bicarbonate Buffer

Vaccine Formulation 4C	Rotavirus antigen 116E target titer of 10^6 FFU/0.5ml, composed of at least 2 Sugars (sucrose 50% and trehalose 0.5%) Lactalbumin hydrolysate 0.5%. (Sample 8 below).
Vaccine Formulation 4D	Rotavirus antigen 116E target titer of 10^6 FFU/0.5ml, composed of at least 2 sugars (Sucrose-40%, Trehalose-0.5%), 0.5% lactalbumin hydrolysate, 0.35% human serum albumin and 1.1M Phosphate Buffer.

Table 6: Details of Vaccine Formulations:

Following observations (Table 7) were made for Baby Rosset Rice assay performed with the vaccine formulations 4A to 4D listed above.

Vaccine Formulations	Initial pH	pH after addition of Acid	Volume of 0.1N HCl added	Baby Rosset Rice Assay Values	Initial Vaccine Titers in FFU/0.5 ml	Vaccine Titer in FFU/0.5 ml After 1 hour
Vaccine formulation 4A at -20°C without buffer of 0.5 mL dose.	7.48	1.75	4.0 mL	BRR of less than 0 minutes (immediate drop in pH)	$10^{4.83}$	$10^{4.33}$
Vaccine formulation 4B at -20°C with 2.5 ml of citrate bicarbonate buffer of 0.5 mL dose.	8.35	4.22	10.0 mL	BRR of 12 minutes	$10^{6.17}$	$10^{6.12}$
Vaccine formulation 4C at $5\pm 3^{\circ}\text{C}$ without buffer of 0.5 mL dose.	7.28	3.98	5.0 mL	BRR of 2 minutes	$10^{5.30}$	$10^{5.30}$
Vaccine formulation 4D at $5\pm 3^{\circ}\text{C}$ with buffer of 1.5 mL or 2 mL dose.	6.75	4.33	10.5 mL	BRR of approximately 13 minutes	$10^{6.12}$	$10^{6.12}$

Table 7: Baby Rosset Rice analysis in comparison to rotavirus vaccine formulations with and without buffer.

Conclusion: Therefore from the above table we find that, even if for the vaccine formulation 4A above which shows immediate drop from pH 7.48 to pH 1.75, the vaccine titer is retained from $10^{4.83}$ FFU/0.5 ml and $10^{4.33}$ FFU/0.5 ml. Same way, the vaccine titer is also

retained in case of Vaccine formulation 4C between $10^{5.30}$ to $10^{5.02}$ FFU/0.5 ml when the pH drops from 7.28 to pH of 3.98 within 2 minutes. Therefore, even if the BRR value is only 2 minutes, the vaccine titer is fairly retained i.e. $10^{5.02}$ FFU/0.5 ml. This much amount of vaccine titer is enough for generation of immune response and thereby conferring protection and prevention of rotaviral gastroenteritis in human infants.

Embodiment 4: Clinical trial without Buffer for ROTAVAC® comprising rotavirus 116E strain.

Hypothesis and Study Rationale

Natural transmission of rotavirus is assumed to occur via faecal-oral route. Rotaviruses in general are acid labile and it is believed that acidic environment affects the viability of the virus.

Given their acid-labile nature of rotaviruses, are so efficient in ubiquitously infecting mammals, most of which have gastric pH values around 2. One possibility is that the human infant stomach may be somewhat more permissive for survival of rotavirus than the adult stomach, as infant gastric pH levels tend to be approx. 3.2 compared with adults approximately 1.0. This could account for the fact that 60 to 90% of reported human rotavirus disease occurs in children below the age of 3 years.

Study Design

In order to test this hypothesis a large multicenter randomized controlled trial was undertaken to evaluate and compare the vaccine immune response in subjects receiving ROTAVAC with citrate bicarbonate buffer to those who receive ROTAVAC without the buffer.

Accordingly, the study included the following three treatment groups:

- Group I (received ROTAVAC® with 5 minutes prior administration of 2.5 ml of buffer)
- Group II (received ROTAVAC® without buffer)
- Group III (received ROTAVAC® immediately mixed with 2.5 ml of buffer prior to administration).

The study investigator and safety assessors (study coordinators) were blinded to the treatment group assignment. Blinding was achieved by a study nurse administering the vaccine as per the allocated treatment group. All subjects received 3 doses of the vaccine given 4 weeks apart. Vaccine immune response was tested 4 weeks after the third vaccine dose (day 84) and compared to baseline (day 0). Immunogenicity was tested in terms of Geometric Mean Titres.

(GMTs) of serum anti rotavirus IgA and seroconversion (fold rise in antibody levels from pre vaccination to post vaccination) in the treatment groups.

Study Results and Conclusions

Post vaccination immune response was comparable with no statistically significant difference in the anti rotavirus IgA response between the treatment groups. Importantly, seroconversion and GMTs achieved in group II (ROTAVAC[®] administered without buffer) was similar to group I in the other two treatment groups where ROTAVAC[®] was administered with buffer.

Post vaccination anti-rotavirus IgA GMTs in the group where ROTAVAC was administered without buffer was 20.7U/mL in comparison to 19.6U/mL and 19.2U/mL in the two groups which received antacid buffer 5 minutes prior to vaccine and simultaneously mixed with vaccine respectively (Table 1). The differences between the groups were not statistically significant ($p>0.05$, Student's T-test).

Table 1: Geometric Mean Titres & 95% CI for results (day 0 & day 84)

Visit	Group I (N = 290)		Group II (N = 287)		Group III (N = 286)	
	GMT	95% CI	GMT	95% CI	GMT	95% CI
DAY0	10.5	(9.4, 11.7)	10.8	(9.7, 11.9)	10.2	(9.2, 11.3)
DAY84	19.6	(17.0, 22.7)	20.7	(17.9, 24.0)	19.2	(16.8, 22.1)

N= Number of Subjects evaluated in each group; GMT=Geometric Mean; 95 % CI (LL, UL) = Confidence Intervals (Lower Limit, Upper Limit)

Similarly, the four-fold seroconversion observed in the groups that received ROTAVAC without antacid buffer and with buffer was similar at 29.2%, 24.5% and 25.1% respectively (Table 2). Further, the lower limit of the 95% confidence interval of the difference in seroconversion (2-fold) between the treatment groups was $>-10\%$, indicating non-inferiority in the immune response achieved with all three treatment regimens (table 3).

Table 2: Seroconversion and 95% CI (2, 3 or 4 fold change in antibody titres) between baseline and day 84 post-vaccination

Fold Change	Group I (N = 290)		Group II (N = 287)		Group III (N = 286)	
	%	95% CI	%	95% CI	%	95% CI

2	30.7	(25.7, 36.2)	35.2	(29.9, 40.9)	33.5	(28.3, 39.2)
3	27.6	(22.8, 33.1)	32.4	(27.3, 38.1)	31.8	(26.7, 37.4)
4	24.5	(19.8, 29.7)	29.2	(24.3, 34.8)	25.1	(20.5, 30.5)

N = Number of subjects evaluated in each group; (95 % CI) = Confidence Intervals (Lower Limit, Upper Limit)

Table 3: Difference in % 2-fold seroconversion at post vaccination in the treatment groups

% 2-fold Seroconversion post vaccination (day 84)						
	Group I (N = 290)		Group II (N = 287)		Group III (N = 286)	
	%	95% CI	%	95% CI	%	95% CI
DAY 84	30.7	(25.7, 36.2)	35.2	(29.9, 40.9)	33.5	(28.3, 39.2)
Difference in % seroconversion with 95% CI 2-Sided, Chi-Square Test						
Group	% (95% CI of difference)					
Group I Vs Group II	4.5 (-3.5, 12.5)					
Group II Vs Group III	1.6 (-6.5, 9.7)					
Group I Vs Group III	2.9 (-4.7, 10.5)					

The reactogenicity and safety with respect to solicited and unsolicited adverse events were comparable across the three groups with no statistically significant difference. ROTAVAC® vaccine was well tolerated in all three treatment groups that received the vaccine with or without the antacid buffer.

Embodiment 5: New Rotavirus 116E formulations

Stability data of ORV 116E liquid formulations at 37°C, 25°C and 2-8°C

The rotavirus vaccine formulations of a dose volume of 0.5 ml without bufer (samples 1 to 12, 14 to 16), and with mixed buffer at 0.5 ml dose volume (sample 13) have been examined for prolonged period of time ranging for a period upto 5 years, is provided below. The various formulation details is provided below along with their stabilites at 37°C for upto 4 weeks, 25°C for at least 6 months, and for 5±3 °C for upto 2years to 5 years. The studies establish that, the rotavirus vaccine formulations without buffer at dose volumes of 0.5 ml are also stable in nature for prolonged periods of time at refrigerated temperatures for at least 2 years. Rotavirus vaccine formulations are also found to be stable for a period of 5 years at

refrigerated temperatures as well.

Sample 1 is formulated containing rotavirus 116E, Sucrose 50%, Lactose 0.5%, HSA 0.5% and Lactalbumin hydrolysate 0.05%.

Stability of Sample 1:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M
5±3 °C	6.02	6.0			6.43		6.21	6.16	6.16
25°C	6.02	6.07	5.81	5.9	5.51	4.65			
37°C	6.02	5.32	2.63						

Sample 2 is formulated containing rotavirus 116E, Sucrose 50%, Trehalose 0.5%, HSA 0.5% and Lactalbumin hydrolysate 0.5%.

Stability of Sample 2:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M
5±3 °C	6.09	6.14			6.19		6.07	6.10	6.05
25°C	6.09	6.02	6.06	5.63	5.51	2.48			
37°C	6.09	5.47	3.23						

Sample 3 is formulated containing rotavirus 116E, Sucrose 50%, Trehalose 1.0%, HSA 0.5% and Lactalbumin hydrolysate 0.5%.

Stability of Sample 3:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M
5±3 °C	6.22	6.24			6.26		6.14	6.20	6.13
25°C	6.22	6.18	6.15	5.80	5.34	2.03			
37°C	6.22	5.35	3.22						

Sample 4 is formulated containing rotavirus 116E, Sucrose 40%, Lactose 0.5%, and Lactalbumin hydrolysate 0.05%.

Lactalbumin hydrolysate 1.0%.

Stability of Sample 4:

Temp	0 day	1M	2M	3M	6M	9M	12M	15M	24M	6
5±3 °C	6.19	6.18			6.08		6.21	6.24	6.12	
25°C	6.19	5.66	5.10	4.8	3.01					
37°C	6.19	4.23	2.01							

Sample 5 is formulated containing rotavirus 116E, Sucrose 40%,Maltose5.0% and Lactalbumin hydrolysate 1.0%.

Stability of Sample 5:

Temp	0 day	1M	2M	3M	6M	9M	12M	15M	24M	6
5±3 °C	6.35	6.37			6.18		6.01	6.17	6.08	
25°C	6.35	5.89	5.11	4.77	2.43					
37°C	6.35	5.31	3.58							

Sample 6 is formulated containing rotavirus 116E, sucrose 40%, Trehalose 0.5% and lactalbumin hydrolysate 1%.

Stability of Sample 6:

Temp	0 day	1M	2M	3M	6M	9M	12M	15M	24M	6
5±3 °C	6.19	6.15			6.10		6.03	6.22	6.13	
25°C	6.19	5.87	5.43	5.12	3.25					
37°C	6.19	4.02	2.52							

Sample 7 is formulated containing rotavirus 116E, Sucrose 40%,Trehalose 1.0% and Lactalbumin hydrolysate 1.0%.

Stability of Sample 7:

Temp	0 day	1M	2M	3M	6M	9M	12M	15M	24M
5±3 °C	6.34	5.79			6.00		5.55	6.07	6.09
25°C	6.34	6.07	5.81	5.9	5.51	4.65			
37°C	6.34	5.32	2.63						

Sample 8 is formulated containing rotavirus 116E, sucrose 50%, Trehalose 0.5%, lactalbumin hydrolysate 0.5%.

Stability of Sample 8:

Temp	0 day	1M	2M	3M	6M	15M	18M	24M	36M
5±3 °C	6.34	6.34			6.33	6.31	6.24	6.14	6.08
25°C	6.34	6.32	6.14	6.01	5.25				
37°C	6.34	5.11	4						

Sample 9 is formulated containing rotavirus 116E, Sucrose70%,Trehalose 0.5%.

Stability of Sample 9:

Temp	0 day	1M	2M	3M	6M	15M	18M	24M	36M
5±3 °C	6.38	6.31			6.23	6.44	6.35	6.26	6.18
25°C	6.38	6.41	6.35	6.23	5.57				
37°C	6.38	5.07	4.01						

Sample 10 is formulated containing rotavirus 116E, Sucrose 50%,lactalbumin hydrolysate 0.5%,Maltose0.5%,HSA 0.5% and Lactalbumin hydrolysate 0.05%.

Stability of Sample 10:

Temp	0 day	1M	2M	3M	6M	15M	18M	24M	36M
5±3 °C	6.25	6.15			6.03	6.34	6.08	6.12	6.11

25°C	6.25	6.14	6.27	6.12	5.88				
37°C	6.25	5.02	4.35						

Sample 11 is formulated containing rotavirus 116E, Sucrose 50%,Trehalose 0.5%,malto 0.5%, HSA 0.5%.

Stability of Sample 11:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M
5±3 °C	6.68	6.62	6.95	6.84	6.64	6.65	6.37	6.72	6.05
25°C	6.68	6.50	6.20	6.04	5.32	4.47			
37°C	6.68	6.10	5.58	4.26					

Sample 12 is formulated containing rotavirus 116E, Sucrose 50%,Trehalose 0.5%, HSA 0.5% and Lactalbumin hydrolysate 0.05%.

Stability of Sample 12:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M
5±3 °C	6.62	6.76	6.98	7.00	6.86	6.74	5.99	6.46	6.17
25°C	6.62	6.46	6.42	5.99	5.13	3.25			
37°C	6.62	5.58	5.06						

Sample 13 is formulated containing rotavirus 116E, sucrose (40%), trehalose (0.5%), lactos (5%), rHSA (0.5%), LAH (1%) and mixed buffer (ammonium acetate + ammonium bicarbonate + diammonium orthophosphate).

Stability of Sample 13:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M
5±3 °C	6.61	6.59	6.58	6.60	6.53	6.54	6.18	6.01	5.83
25°C	6.61	6.46	6.44	5.79	5.25	4.15			

37°C	6.61	5.56	5.01						
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Stability of Sample 13 at -20oC:

Temp	0 day	1M	2M	3M	6M	9M	12M	24M	36M
-20°C	6.61	6.59	6.59	6.56	6.54	6.52	6.21	6.11	6.13

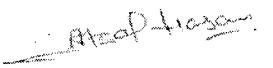
Sample 14, 15 and 16 are formulated with rotavirus 116E, 10% SPG dissolved in DME. The samples 14, 15, and 16 are kept at -20°C, and stability checked for 60 months. vaccine formulations 13, 14, and 15 are found to be very stable at -20°C for a period of at least 5 years.

Vaccine sample	0 Day	3M	6M	9M	12M	24M	36M	48M	60M
Sample 14 (10 ^{4.0} FFU/0.5 mL)	4.25	4.01	4.08	4.15	4.25	4.02	4.18	4.12	4.08
Sample 15 (10 ^{5.0} FFU/0.5 mL)	5.55	5.42	5.68	5.37	5.77	5.89	5.32	5.40	5.32
Sample 16 (10 ^{6.0} FFU/0.5 mL)	6.12	6.12	6.06	6.33	6.36	6.27	6.15	6.11	6.05

Thus, it is established that various rotavirus vaccine compositions of 0.5 ml of dose volume are stable at -20°C for 5 years, 2-8°C for 2 years, 25°C for 6 months, and 37°C for 1 week. Irrespective of the vaccine formulation, the rotavirus vaccine at 0.5 ml to 1 ml dose volume is also found to be acid stable, as exemplified in embodiment 3 as well at a pH range of 2 to 4. Therefore, applicants wish to mention that irrespective of the vaccine composition details, any rotavirus vaccine comprising the live attenuated rotavirus 116E is capable to withstand the strong acidic environment in the human infant stomach and produce the required immune response against the rotavirus infections. The vaccine formulations presented in this patent application are only meant for the purposes of explaining the breadth and depth of the invention. It is impractical to provide all possible vaccine formulation details by the applicant, although the applicant has given numerous formulation strategies in Embodiment 3, Example 3 and Embodiment 5. Therefore, all possible obvious variations by a person skilled in the art for rotavirus vaccine formulation strategy comprising the rotavirus 116E strain

also covered under this invention. It is to be understood and accordingly construed that, the vaccine compositions which are not specifically included in this patent application comprising the live attenuated rotavirus 116E is also covered under the scope of this invention.

Dated this 16th day of June 2016


Afzal Hasan
of HASAN AND SINGH

ABSTRACT

A BUFFER FREE, ACID STABLE, LOW DOSE VOLUME ROTAVIRUS VACCINE

A buffer free, acid stable, low dose volume rotavirus vaccine is disclosed. The vaccine is available in dose volume of less than 1 ml per dose for oral administration and it is without buffer. The vaccine also does not require pre or post administration of any antacid at the time of oral administration of the vaccine to the subject to neutralize the stomach acid. The vaccine exemplifies nominal drop in vaccine titer at pH 2-4 for a time span of 30 minutes. The vaccine is stable at -20°C for at least 60 months.