In one of the embodiment, the methodology of Design-of-Experiment (DoE) is employed for identification of the potential excipients, their concentrations and combinations thereof that are likely to have a significant impact on the potency loss of viruses in the vaccine formulation during freeze-drying process and other manufacturing steps and subsequent storage for determining the stability loss. In some cases, to minimize number of experiments, successive fractional factorial DoE have also been employed.

The suitability of potential excipients, their concentrations and combinations thereof is determined by various biophysical methods, for example, Differential Thermal Analysis (DTA), Differential Scanning Calorimetry (DSC), Freeze Drying Microscopy (FDM) and Impendence measurements . Further, direct potency is also measured by using various methods,for example, Multivalent QPCR-Based Potency Assay (M-QPA) that permits the specific quantitation of each individual reassortant virus in the presence of the other four reassortant viruses. The biophysical methods give an indication of process suitability such as e.g. time and energy inputs required and the latter potency measurement methods gives an indication of the effect of excipients including stabilizers on the potency during processing and subsequently during storage. In some cases, potency change after repeated freeze-thawing of the virus in presence of DoE compositions followed by incubation at elevated temperature i.e. at 25°C for extended period of time for at least 24 hours and a maximum of up to one week have also been used.

The excipients including various stabilizers are selected from, but not limited to (a) lyoprotectants such as Sucrose, Trehalose, Sorbitol and Lactose, (b) divalent cation more particularly divalent ionic structural stabilizers such as Zn (II) and Ca (II); (c) buffering agents such as HEPES, Tris, Phosphate, Citrate or Histidine; and (d) salts such as Sodium Chloride (NaCl), Magnesium chloride, Potassium chloride; (e) bulking agents such as Polyvinylpyrrolidone(PVP), Dextran,Mannitol; (f) activating agents including L-Arginine and / or Glycine and (g) dispersants such as Tween (e.g. Tween® 20 and Tween® 80).

associated with presence of water. The said rotavirus vaccine preparations can be obtained using any appropriate freeze drying method.

In a preferred embodiment, freeze drying formulation ingredients in composition and concentration are selected based on Potency and biophysical measurements to arrive at a formulation affording optimal freeze drying parameters and times with no more than Log_{10}=[0.2] process loss for the said virus serotypes.

In yet another embodiment, said rotavirus vaccine formulation can be freeze dried in vials in a monodose and/or multidose formulations and the said vaccine is reconstituted with the provided reconstitution buffer prior to administration.

In yet another embodiment, the said rotavirus vaccine formulation is freeze dried in bulk using commercially available freeze drying trays like GORE® LYOGUARD® Freeze-Drying Tray or stainless steel trays.

In yet another preferred embodiment, said rotavirus vaccine formulation is freeze dried in bulk for example rotavirus vaccine with 5-10 fold higher titers than the clinically relevant specifications are freeze dried thus reducing both the size and number of batches and thereby reducing the overall cost of production.

In order to obtain a powder suitable for filling in the container closure for particularly monodose container, the bulk freeze dried material is milled resulting in the milled freeze dried powder with particle size of D_{90} value not less than 250 µm. The milling unit process can be accomplished using appropriate milling methods including but not limited to Sieve milling, Hammer milling, Conical sieve Milling, Ball milling and Roller milling at temperatures ranging from 0°C to 25°C.

In yet another embodiment, the milled freeze dried powder can be blended with suitable blending agents selected from but not limited to PVP K25, PVP K40, sucrose, mannitol, maltodextrin with dextrose equivalent (DE) generally ranging from 4 to 20, fructose,

glucose, lactose or placebo formulation of same composition or the combinations thereof. The said combinations are so prepared to obtain a powdered dosage form having rotavirus titers equal to clinically relevant minimal concentrations or any desired release specification as well as to engineer powder properties acceptable for accurate and precise powder filling in the predetermined packaging containers.

In yet another embodiment, the freeze drying is performed either at clinically relevant specification or at higher titre of rotavirus scales (5x in glass vials or in bulk using stainless steel trays with a process loss of not more than Log_{10}=[0.2], cumulative potency (process and stability) losses for the freeze dried rotavirus vaccine serotype in any combination do not exceed Log_{10}=0.5 irrespective of factors such as starting fill, serotype, process scale, and combinations thereof at 37^{0}C±2 ^{0}C (RH 75% ±5.0%) for at least 30, 90 or 180 days and a cumulative potency (process and stability) losses for the freeze dried rotavirus vaccine serotype in any combination do not exceed Log_{10}=0.5 irrespective of the factors irrespective of factors such as starting fill, serotype, process scale, and combinations thereof at 45^{0}C±2 ^{0}C (RH 75% ±5.0%) for at least 30, 90 or 120 days.

Rotavirus is acid-labile and gets rapidly inactivated with a half-life of less than 12 minutes at pH 2.0. The rotavirus vaccines are intended to be administered orally, it is crucial to protect rotavirus which may get inactivated by gastric acid present in stomach. The effective way for administering such vaccines is to use antacids or neutralizing buffers before or in combination with the oral vaccine.

In yet another embodiment of the present invention, special provisions are employed to protect the vaccine from stomach acid after oral administration, stomach acid neutralization is accomplished by administering the vaccine with buffering agent with better Acid Neutralization Capacity (ANC). The buffering/ antacid component are selected from but not limited to HEPES, Trisodium citrate dihydrate, histidine, calcium carbonate, sodium carbonate, potassium carbonate,

sodium bicarbonate, calcium bicarbonate, potassium bicarbonate, aluminum hydroxide, sodium dihydrogen phosphate monohydrate or magnesium hydroxide or combinations thereof.

In yet another embodiment of the present invention, said vaccine formulation is milled and and blended with blending agent and/or buffering agent imparting desired ANC which is in the range of 0.3 mEq/ dose to 1.0 mEq/dose of vaccine powder and moisture content is below 3%.

In yet another embodiment of the present invention, said vaccine formulation is reconstituted with a reconstitution buffer just before oral administration that imparts desired ANC which is in the range of 0.3 mEq/dose to 1.0 mEq/dose of vaccine.

In another embodiment, to provide the palatability to the formulation, sugars are added in the concentration in the range of 0% w/v to 60% w/v to reconstitution buffer. The sugars are selected from but not limited to lactose, glucose, sucrose, fructose or the combinations thereof.

In all aspects of the present invention vaccine formulations are in dry form i.e. Freeze dried cake/powder, milled dried powder or milled and blended freeze dried powder. The said above formulations so obtained do not exceed potency losses exceeding Log10 = [0.5] when stored at 5 °C ± 3°C for at least 9 months that may be statistically extrapolated to 2 years) and at 37°C or 45°C for at least 18 weeks.

In all aspects of the present invention said powdered vaccine formulations are administered orally by reconstituting with either said reconstitution buffer when blended solely with blending agent or with water for Injection when blended with blending agent and with buffering agents having desired ANC.

Yet another aspect of the present invention provides aseptically reconstituted vaccine preparation that does not result in overall (process + stability) virus potency losses exceeding Log10 = [0.5] when re-constituted in appropriate aqueous reconstitution buffer and stored at 2°C - 8°C for 48 h.

In yet another embodiment of the present invention, the glass transition temperature (Tg) of milled vaccine is more than 47°C to provide the thermal stability at higher temperature storage conditions.

In yet another embodiment, the powdered vaccine formulation is dispensed in the final packaging container/closures including sachet or vials supplied with separate reconstitution buffer for reconstitution.

Thus, said vaccine formulation have improved heat- stability, easy to transport and use and is affordable, hence said vaccine meets requirements of developing and low income country's vaccination program.

EXAMPLES

The following examples illustrate but do not limit the scope of this invention.

Example 1: Thermostabilization of rotavirus serotypes using freeze drying process

Study 1: Selection of Excipients including Stabilizers:

To identify among the potential ingredients, their concentrations and combinations thereof that are likely to have a significant impact on the stability of rotavirus during freeze-drying process including further processing steps (process loss) and storage (stability loss) as well as to support optimal and economical freeze-drying process, the methodology of Design-of-Experiment (DoE) is applied. The G1 strain of rotavirus is used as representative strain to report on the virus potency changes. To minimize number of experiments, successive fractional factorial designs are used. The fractional factorial designs are limited to the 8 factors as shown in the **Table 1** and **Table 2.**

Table 1: Various stabilizers and their concentrations used to design DoE formulations

Buffer		NaCl	Sugar/ Bulking Agent	Amino-acids		Divalent cations		Dispersant
Factor	1	2	3	4	5	6	7	8
Excipient	Tris/ Phosphate /HEPES (mM)	NaCl (mM)	Sugar/ (mannitol or dextran or PVP)	L-Arginine (mM)	Glycine (mM)	$CaCl_2.2H_2O$ (mM)	$ZnCl_2$ (mM)	Tween (%)
Low level (-1)	10	0	1.2/2	10	0	0	0	0
Mid level (0)	25	25	3/3	50	50	1	1	0.01
High level (1)	50	50	4/4.8	100	100	2	2	0.02

Table 2: Combinations of buffer and bulking agents used to design DoE formulations

Combination	1	2	3	4	5	6	7	8	9
Buffer	Phosphate	Phosphate	Phosphate	HEPES	HEPES	HEPES	Tris	Tris	Tris
Bulking agent	Dextran	PVP	Mannitol	Dextran	PVP	Mannitol	Dextran	PVP	Mannitol

To further simplify the screening design, one buffer and one combination of sugar and bulking agent at a time is selected in a step by step approach. This fractional factorial design yielded total 19 experiments per combination wherein the concentration of buffering agent, salt, sugar: bulking agent ratio, amino acids, activating agent and dispersant are varied. To test the ability of the stabilizer combination for optimal freeze-drying process design and for supporting virus stability during freeze-drying, following screening experiments are conducted:

WE CLAIM:

1. A thermostable freeze dried rotavirus vaccine formulation wherein said vaccine formulation comprises of
   - at least one rotavirus serotype, and
   - at least one excipient comprising of a lyoprotectant, a first class of buffering agent, a salt, an ion, a bulking agent, a dispersant, an activating agent, either alone or in combination thereof.

2. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein
   - said lyoprotectant is selected from sucrose, trehalose, sorbitol or lactose;
   - said first class of buffering agent is selected from HEPES, Tris, Phosphate, citrate or Histidine;
   - said salt is selected from Sodium chloride, Magnesium chloride, Potassium chloride;
   - said ion is a divalent cation selected from Zn (II) and Ca (II);
   - said bulking agent is selected from Polyvinylpyrrolidone (PVP), Dextran or Mannitol;
   - said dispersant is selected from Tween® 20 and Tween® 80;
   - said activating agent is selected from L-Arginine, Glycine; either alone or in combination.

3. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein
   - said lyoprotectant is in the range of 1.2% w/v to 6.0% w/v;
   - said first class of buffering agent is in the range of 10 mM to 50 mM;
   - said salt is in the range of 0mM-50mM;
   - said ion is in the range of 0 mM to 2 mM;
   - said bulking agent is in the range of 2% to 6 % w/v;

- said dispersant is in the range of 0 to 0.02%w/v;
- said activating agent is in the range of 10 to 100mM.

4. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 and claim 3 wherein
   - said lyoprotectant is Sucrose, Trehalose or combination thereof
   - said first class buffering agent is HEPES;
   - said salt is Sodium Chloride (NaCl);
   - said divalent cation is Ca^{+2};
   - said first class of bulking agent is PVP;
   - said dispersant is Tween® 20; and
   - said activating agent is L-Arginine and/or Glycine.

5. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein said rotavirus serotype is selected from at least one rotavirus strains selected from bovine, rhesus, human, ovine, rhesus/human reassortants, bovine/human reassortants and other monovalent or multivalent rotavirus.

6. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein said rotavirus serotype is selected from G1, G2, G3, G4 and P1 Human- Bovine reassortants either alone or in combination.

7. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 wherein said rotavirus serotype having Log10 concentration as G1: 6.81, G2: 6.92, G3: 6.91, G4: 6.78 and P1: 6.83 per dose.

8. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein said vaccine formulation does not exceed potency losses exceeding Log_{10} = [0.5] when stored at 5 ± 3°C up to 2 years and when stored up to 45°C for at least 18 weeks.

9. An improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1, said process comprising the steps of:

(a) selecting at least one rotavirus serotype;

(b) adding said selected rotavirus serotype of step (a) to said at least one excipient to prepare a liquid feed with optimal potency retention;

(c) freeze drying said aqueous liquid feed of step (b) to obtain a freeze dried formulation with reduced residual moisture content;

(d) milling said freeze dried formulation obtained from step (c) by using suitable milling methods at temperatures ranging from 0°C to 25°C to obtain a milled dried formulation; and

(e) blending of said milled dried formulation obtained from step (d) with a blending agent individually or in combination with a second class of buffering agent having acid neutralizing capacity to obtain said thermostable freeze dried rotavirus vaccine formulation for appropriate packaging of monodose or multidose,

-wherein the said process result in a process loss not exceeding Log_{10} = [0.2].

10. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said liquid feed is having solid contents in the range of 10g/100ml to 25g/100ml, more preferably 13g/100ml (13%).

11. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said freeze drying process is performed either at clinically relevant specification (1x) or at higher (5x) rotavirus concentrations.

12. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said liquid feed is having titre value in the range of 5.0E+06 IU/mL to 6.0E+07 IU/ mL each serotype, preferably 5.0E+06 IU/mL to 4.0E+07 IU/mL each serotype.

13. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said freeze drying process results in cumulative potency loss not exceeding Log_{10} = [0.5] at temperature in the range of 37°C ± 2°C for at least 30, 60, 90, 180 days and Log_{10} = [0.5] at temperature in the range of 45°C ± 2°C for at least 30, 60, 90, 120 days.

14. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said milled freeze dried powder is obtained by milling methods including Sieve milling, Hammer milling, Ball milling, Conical sieve milling and Roller milling etc. wherein;

- Particle size of said milled freeze dried powder have D_{90} value, is not less than 250 µm;
- the preferred temperature range is in range of 20°C - 22°C; and
- said milling is performed in presence of a dry gas including Nitrogen, Argon, CO_2, air or combinations thereof.

15. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said vaccine formulation is in dry form such as freeze dried cake, freeze dried powder, milled freeze dried powder, milled and blended freeze dried powder.

16. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 14 wherein said milled

freeze dried powder shows glass transition temperature (T_g) in the range of 48.0 ± 1.0°C.

17. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said residual moisture content of said freeze dried rotavirus vaccine formulation is below 3.0 %.

18. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said blending agent is selected from PVP K25, PVP K40, sucrose, mannitol, maltodextrin with DE ranging from 4 to 20, fructose, glucose, lactose either alone or in the combinations providing D_{90}> 250 µm to milled blended freeze dried powder.

19. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said second class of buffering agent being selected from HEPES, Trisodium citrate dihydrate, histidine, calcium carbonate, sodium carbonate, potassium carbonate, sodium bicarbonate, calcium bicarbonate, potassium bicarbonate, aluminum hydroxide, sodium dihydrogen phosphate monohydrate or magnesium hydroxide or combinations thereof.

20. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said second class of buffering agent when used in conjunction with blending agent results in said acid neutralization capacity of the vaccine in the range of 0.3 mEq/dose to 1.0 mEq/dose of powder.

21. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 9 wherein said vaccine is

to be administered orally with or without a reconstitution buffer or with or without water for injection.

22. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 21 wherein said reconstitution buffer is selected from calcium carbonate, sodium carbonate, potassium carbonate, sodium bicarbonate, calcium bicarbonate, potassium bicarbonate, aluminum hydroxide or magnesium hydroxide, sodium dihydrogen phosphate monohydrate, trisodium citrate dihydrate or combinations thereof, more preferably phosphate-citrate.

23. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 22 wherein said vaccine formulation when reconstituted with reconstitution buffer results in acid neutralizing capacity in the range of 0.3 mEq/dose to 1.0 mEq/dose.

24. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 23 wherein the palatability of said reconstitution buffer is maintained by adding 0- 60% of sugars such as lactose, glucose, sucrose, fructose or the combinations thereof.

25. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 22 prepared by an improved freeze drying process, said process comprising the steps of:

(a) selecting at least one rotavirus serotype;

(b) adding said selected rotavirus serotype of step (a) to said at least one excipient to prepare a liquid feed with optimal potency retention;

(c) freeze drying said liquid feed of step (b) to obtain a freeze dried formulation with reduced residual moisture content;

(d) milling said freeze dried formulation obtained from step (c) by using suitable milling methods at temperatures ranging from 0°C to 25°C to obtain a milled dried formulation;

(e) blending said milled dried formulation obtained from step (d) with a blending agent individually or in combination with a second class of buffering agent having acid neutralizing capacity to obtain said thermostable freeze dried rotavirus vaccine formulation for appropriate packaging of monodose or multidose; and

(f) packaging of said thermostable freeze dried rotavirus vaccine formulation in a container along with said reconstitution buffer comprising said buffering agent having said acid neutralizing capacity in a separate container for reconstitution prior to oral administration.

26. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 22 wherein said vaccine when reconstituted with said reconstitution buffer maintains virus potency losses not exceeding Log10 = [0.5] at storage conditions of 2°C - 8°C for 48 h and 25°C for 8 h.

27. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein said vaccine formulation dissolves within 60 ± 5.0 sec in said reconstitution buffer maintained at 23°C ± 1°C.

28. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein said formulation is obtained by a process comprising the steps of:

(a) selecting at least one rotavirus serotype;

(b) adding said selected rotavirus serotype of step (a) to said at least one excipient to prepare a liquid feed with optimal potency

retention;

(c) freeze drying said aqueous liquid feed of step (b) to obtain a freeze dried formulation with reduced residual moisture content

-wherein step (c) is performed in vials.

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