VACCINE

TECHNICAL FIELD

This invention relates to rotavirus vaccine formulations. The invention relates to the use of an attenuated rotavirus population from one rotavirus type in the prevention of disease associated with rotavirus infection from another rotavirus type.

TECHNICAL BACKGROUND

Acute, infectious diarrhoea is a leading cause of disease and death in many areas of the world. In developing countries, the impact of diarrhoeal disease is staggering. For Asia, Africa and Latin America, it has been estimated that there are between 3-4 billion cases of diarrhoea each year and of those cases about 5-10 million result in death (Walsh, J.A. et al.: N. Engl. J. Med., 301:967-974 (1979)).

Rotaviruses have been recognised as one of the most important causes of severe diarrhoea in infants and young children (Estes, M.K. Rotaviruses and Their Replication in Fields Virology, Third Edition, edited by Fields et al., Raven Publishers, Philadelphia, 1996). It is estimated that rotavirus disease is responsible for over one million deaths annually. Rotavirus-induced illness most commonly affects children between 6 and 24 months of age, and the peak prevalence of the disease generally occurs during the cooler months in temperate climates, and year-round in tropical areas. Rotaviruses are typically transmitted from person to person by the faecal-oral route with an incubation period of from about 1 to about 3 days. Unlike infection in the 6-month to 24-month age group, neonates are generally asymptomatic or have only mild disease. In contrast to the severe disease normally encountered in young children, most adults are protected as a result of previous rotavirus infection so most adult infections are mild or asymptomatic (Offit, P.A. et al. Comp. Ther., 8(8):21-26, 1982).

Rotaviruses are generally spherical, and their name is derived from their distinctive outer and inner or double-shelled capsid structure. Typically, the double-shelled capsid structure of a rotavirus surrounds an inner protein shell or core that contains the genome. The genome of a rotavirus is composed of 11 segments of double-stranded

RNA which encode at least 11 distinct viral proteins. Two of these viral proteins designated as VP4 and VP7 are arranged on the exterior of the double-shelled capsid structure. The inner capsid of the rotavirus presents one protein, which is the rotavirus protein designated VP6. The relative importance of these three particular rotaviral proteins in eliciting the immune response that follows rotavirus infection is not yet clear. Nevertheless, the VP6 protein determines the group and subgroup antigen, and VP4 and VP7 proteins are the determinants of serotype (types determined by neutralisation assay) and genotype (types determined by a non-serological assay) specificity. The designations for G serotypes and G genotypes are identical. In contrast, the numbers assigned for P serotypes and genotypes are different (Santos N. et Hoshino Y., 2005, Reviews in Medical Virology, 15, 29-56). Therefore the P serotype is designated as P followed by assigned number, and the P genotype is designated by a P followed by assigned number in brackets.

To date, at least 14 rotavirus G serotypes and 14 rotavirus P serotypes have been identified (Santos N. et Hoshino Y., 2005, Reviews in Medical Virology, 15, 29-56). Among these, 10 G (G1-6, G8-10 and G12) serotypes and 9 P (P1, P2A, P3, P4, P5A, P7, P8, P11 and P12) serotypes have been identified among the human rotavirus. Twenty-three P genotypes have been described ten of which have been recovered from humans (P[3]-[6], P[8]-[11], P[14] and P[19].

VP7 protein is a 38,000 MW glycoprotein (34,000 MW when non-glycosylated) which is the translational product of genomic segment 7, 8 or 9, depending on the strain. This protein stimulates formation of the neutralising antibody following rotavirus infection. VP4 protein is a non-glycosylated protein of approximately 88,000 MW which is the translational product of genomic segment 4. This protein also stimulates neutralising antibody following rotavirus infection.

Since VP4 and VP7 proteins are the viral proteins against which neutralising antibodies are directed, they are believed to be prime candidates for development of rotavirus vaccines, affording protection against rotavirus illness.

Natural rotavirus infection during early childhood is known to elicit protective immunity.

A live attenuated rotavirus vaccine is thus highly desirable. Suitably this should be an oral vaccine, as this is the natural route of infection of the virus.

Early vaccine development for preventing rotavirus infections began in the 1970s after the discovery of the virus. Initially, attenuated strains from animals and humans were studied and had mixed or disappointing results. More recent efforts have focused on human-animal reassortants that have been more successful.

A rotavirus strain known as 89-12 has been described by Ward; see US Patent Number 5,474,773 and Bernstein, D.L. et al, Vaccine, 16 (4), 381-387, 1998. The 89-12 strain was isolated from a stool specimen collected from a 14 month-old child with natural rotavirus illness in 1988. According to US Patent Number 5,474,773 the HRV 89-12 human rotavirus was then culture-adapted by 2 passages in primary African Green Monkey Kidney (AGMK) cells and 4 passages in MA-104 cells as described by Ward in J. Clin. Microbiol., 19, 748-753, 1984. It was then plaque purified 3 times in MA-104 cells (to passage 9) and grown after 2 additional passages in these cells. One additional passage was made (passage 12) for deposition with the ATCC under the accession number ATCC VR 2272. The deposited strain is known as 89-12C2.

The 1998 paper in Vaccine by Bernstein et al is referred to below as the Vaccine (1998) paper. The paper describes the safety and immunogenicity of an orally administered live human rotavirus vaccine candidate. This vaccine was obtained from strain 89-12, attenuated by passaging without plaque purification 26 times in primary AGMK cells and then another 7 times in an established AGMK cell line (33 passages in total).

Hereinafter the aforesaid material which has been serially passaged 26 times will be referred to as P26 and the material which has been serially passaged 33 times will be referred to as P33. In general, rotavirus derived by passaging 89-12 n times will be referred to as Pn.

In the examples which follow the P33 material was passaged a further 5 times on Vero cells. This is referred to as P38.

The P26 and P33 isolates described in the Vaccine (1998) paper were not deposited in a culture collection, nor were they analysed to establish their genetic characterisation.

It has now been found that the P26 population described in the literature comprises a mixture of variants. This has been established by genetic characterisation as described hereinbelow (see examples). P26 is therefore not a reliably consistent population for further passages, in particular for the production of vaccine lots. Similarly, P33 comprises a mixture of variants and is not reliably consistent for the production of vaccine lots.

It has been found that the P26 material is a mixture of at least three VP4 gene variants. P33 and P38 are similarly a mixture of two variants. These variants appear to be antigenically different, in terms of neutralising epitopes, to the 89-12C2 strain deposited at the ATCC when evaluating the neutralizing antibody titers of sera from infants vaccinated with P33 against these variants.

Furthermore it has been found that when the P33 material is administered to infants, two identified variants are replicated and excreted. Of 100 vaccinated infants, only 2 showed signs of gastro-enteritis due to rotavirus infection, while 20% of a placebo group were infected. These findings suggest that the identified variants are associated with protection from rotavirus disease.

WO 01/12797 discloses a method of separating rotavirus variants and an improved live attenuated rotavirus vaccine derived from a cloned (homogeneous) human rotavirus strain. Also disclosed is an attenuated rotavirus population (isolate), characterised in that it comprises a single variant or substantially a single variant, said variant defined by the nucleotide sequence encoding at least one of the major viral proteins designated as VP4 and VP7. Protective efficacy of such an oral attenuated human rotavirus vaccine against G9 heterologous strain has been reported in Latin American infants (Perez et al. 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC 2002) 27-30 September 2002, San Diego). WO 05/021033 discloses that one rotavirus serotype may be used to protect against disease caused by another serotype. In particular WO 05/021033 discloses the use of a G1 rotavirus population, [for example as deposited at the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and

Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom on 13 August 1999 under the deposition number 99081301, under the terms of the Budapest Treaty, also named P43 or RIX4414], to prevent disease caused by both G1 and at least one non-G1 rotavirus serotype, such as but not limited to the G2, G3, G4 and G9 rotavirus serotypes.

The whole content of WO 01/12797 and WO 05/021033 is herein incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A (SEQ ID NO:1) is the nucleotide sequence of P43 (RIX4414) VP4 gene including the sequence encoding the VP4 protein of P43.

Figure 1B (SEQ ID NO:2) has additional nucleotides from both ends of the gene and a nucleotide substitution (in bold – a G instead of a C at position 18 with resulting in TCG instead of TCA with however no impact on the resulting encoded protein) due to the sequencing technique. The non-coding sequence appears in small case. Figure 1B shows the correct sequence for the P43 deposit.

Figure 2A (SEQ ID NO:3) is the nucleotide sequence of P43 (RIX4414) VP7 gene including the sequence encoding the VP7 protein of P43.

Figure 2B (SEQ ID NO:4) has additional nucleotides from both ends of the gene and a nucleotide substitution (in bold – a A instead of a C at position 58, resulting in a ATT coding for leucine instead of CTT coding for isoleucine) due to the sequencing technique. The non-coding sequence appears in small case. Figure 2B shows the correct sequence for the P43 deposit.

Figure 3 (SEQ ID NO:5) is the polypeptide sequence of RIX4414 VP4.

Figure 4 (SEQ ID NO:6) is the polypeptide sequence of RIX4414 VP7.

Figure 5 (SEQ ID NO:7) shows the polypeptide sequence of NSP4 protein of RIX4414.

Figure 6 (SEQ ID NO:8) shows the nucleotide sequence encoding NSP4 protein of RIX4414. The non-coding sequence appears in small case.

Figure 7 (SEQ ID NO:9) shows the polypeptide sequence of VP6 protein of RIX4414.

Figure 8 (SEQ ID NO:10) shows the nucleotide sequence encoding VP6 protein of RIX4414. The non-coding sequence appears in small case.

DETAILED DESCRIPTION OF THE INVENTION

In the present invention we have determined that an attenuated rotavirus population, for example one such as characterised in WO 01/12797, can be used as a vaccine to provide cross protection against disease caused by rotavirus infection of a different type (serotype and/or genotype) to that used in the vaccine. The VP7 protein specifies the G type (serotype), and the VP4 protein specifies the P type of strain (serotype or genotype).

In particular the present invention relates to the use of an attenuated rotavirus population from one P type in the prevention of disease associated with rotavirus infection from a different P type, and specifically to the use of an attenuated rotavirus population or strain from a GxPy type in the induction of an immune response and/or in the prevention of disease associated with rotavirus infection caused by a rotavirus strain which is neither a Gx nor a Py type.

Immunity may be measured by neutralising antibody responses to the vaccine or by serum rotavirus IgA antibody response, such as seroconversion factor (i.e. ≥3-fold increase in serum antibody IgA levels following vaccination, as described in Ward et al., 1990, J. Infect. Disease, 161, 440-445).

In the context of this invention, and consistent with the common understanding in the art (Santos N. et Hoshino Y., 2005, Reviews in Medical Virology, 15, 29-56), Gx will refer to a specific G type, i.e. G genotype or G serotype (both terminologies being identical), whilst Py terminology will generically refer to a specific P type, either P serotype (e.g. P8, P4) or P genotype (e.g. P[4], P[8]). When referred to a specific P genotype, the P followed by assigned number in brackets will be used; otherwise P type will mean either serotype or genotype.

Throughout this specification, wording such as the use of a vaccine composition according to the invention in the manufacture of a vaccine composition for the prevention of rotavirus diseases, or such as methods of therapy comprising the use of said vaccine composition will be interchangeably used.

We have now determined that a GxP[8] rotavirus population [for example G1P[8] as deposited at the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom on 13 August 1999 under the deposition number 99081301, under the terms of the Budapest Treaty], can be used to prevent disease caused by both a GxP[8] (e.g. G1P[8]) and at least one rotavirus strain which is neither a Gx nor a Py type. In particular we have determined that a G1P[8] rotavirus population can be used to prevent disease caused by both one G1P[8] and at least one non- G1P[8] genotypes, such as G2P[4] rotavirus genotype.

Accordingly the present invention relates to use of an attenuated rotavirus population from one rotavirus type in the prevention of disease associated with rotavirus infection from another rotavirus type, wherein the type is suitably defined by reference to the sequence of the rotavirus VP4 protein (P type).

The invention also relates to the use of an attenuated rotavirus population from one rotavirus strain (defined by both a specific G and P type) in the prevention of disease associated with rotavirus infection from another rotavirus strain, wherein the strain is suitably defined by reference to the sequence of both the rotavirus VP4 protein (P type) and VP7 protein (G type). Specifically the present invention relates to the use of an attenuated rotavirus strain from a GxPy type in the manufacture of a medicament for inducing an immune response against rotavirus infection caused by a rotavirus strain which is neither a Gx nor a Py type. In other words, a rotavirus strain of the invention can be used to prevent disease caused by infection of a second rotavirus which differs in both the G and P type.

In particular, in all aspects of the claimed invention said immune response is a protective immune response. Suitably the rotavirus population comprises VP4 and/or VP7 viral proteins from ECACC deposit 99081301 suitable to provide a cross protective effect.

Throughout the document, it will be referred to cross-protection as being the protection afforded by a rotavirus type against infection caused by a rotavirus of a different type.

Cross-protection can be homotypic or heterotypic. Homotypic cross-protection is a protection afforded by a rotavirus strain against a strain of either a G or a P type, such as for example a G1P[8] strain affording cross-protection against a non-G1, P[8] strain (e.g. G2P[8]) via the P[8] type. Another example of a homotypic cross-protection is that afforded by a G1P[8] strain against a G1 non-P[8] strain (e.g. G1P[4]) via the G1 type. Heterotypic cross-protection is a protection afforded by a rotavirus strain against a rotavirus strain of different P and G types such as for example the protection afforded by a G1P[8] against a non G1- non P[8]- strain (e.g. G2P[4]) (heterotypic protection afforded via both G and P types).

Suitably the attenuated rotavirus serotype is G1 and is able to provide cross protection against disease caused by G1 and non-G1 rotavirus serotypes such as serotypes selected from the group consisting of: G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13 and G14.

In particular the use of a G1 attenuated rotavirus population, [for example as deposited at the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom on 13 August 1999 under the deposition number 99081301, under the terms of the Budapest Treaty], can be used to prevent disease caused by G1 and at least one, suitably at least two, suitably at least three, suitably at least four non-G1 rotavirus serotypes selected from the group consisting of: G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13 and G14. Accordingly, there is provided the use of an attenuated rotavirus strain from a G1 type in the manufacture of a vaccine composition for the induction of an immune response against a rotavirus infection caused by a rotavirus strain which is not from a G1 type. In a particular aspect, an immune response is induced against at least one, at least two or more rotavirus non-G1 serotypes, typically against any serotype selected from the group consisting of: G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13 and G14. Typically an immune response is induced against at least one, suitably at least two, suitably at least three of the following non-G1 types: G2, G3, G4 and G9, in addition to homotypic (G1) protection. Suitably the composition comprises a G1 rotavirus strain and is used to induce an immune response to the G1 and G2 types.

Suitably the rotavirus attenuated strain type is P[8] and is able to provide cross-protection against disease caused by P[8] rotavirus type and by non-P[8] rotavirus types such as types selected from the group consisting of: P[1], P[2], P[3], P[4], P[5], P[6], P[7], P[9], P[10], P[11], P[12] , P[14] and P[19].

In particular the use of a P[8] attenuated rotavirus population, [for example as deposited at the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom on 13 August 1999 under the deposition number 99081301, under the terms of the Budapest Treaty], can be used to prevent disease caused by P[8] and at least one of the non- P[8] rotavirus types, selected from the group consisting of: P[1], P[2], P[3], P[4], P[5], P[6], P[7], P[9], P[10], P[11], P[12] , P[14] and P[19]. In particular an immune response is suitably induced against at least a P[4] type in addition to the P[8] rotavirus type.

Suitably the vaccine composition for use according to the invention comprises a G1P[8] rotavirus strain and is capable of inducing an immune response to a G2P[4] rotavirus strain.

In a particular aspect, the invention relates to a method of inducing an immune response against rotavirus strain, the method comprising administering to a subject a composition comprising an attenuated rotavirus strain of a GxPy type, said composition generating an immune response against a rotavirus strain which is neither a Gx nor a Py type.

In particular, the invention relates to a method of inducing an immune response against rotavirus G1 and non- G1 serotype, the method comprising administering to a subject a composition comprising a rotavirus G1 serotype vaccine. Suitably non-G1 rotavirus serotypes are selected from the group consisting of: G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13 and G14. Suitably the composition comprises a G1 rotavirus strain and is used to induce an immune response to the G1 and G2 types.

Suitably the vaccine composition for use according to the invention comprises a G1P[8] rotavirus strain and is capable of inducing an immune response to a G2P[4] rotavirus strain.

Suitably the rotavirus population within the vaccine composition is of G1P1A (i.e. G1P[8] according to the current nomenclature) strain specificity. Suitably the rotavirus population comprises VP4 and/or VP7 viral proteins from ECACC deposit 99081301 suitable to elicit an immune response and, typically, provide a cross protective effect. Suitably the invention relates to G1P[8] rotavirus strains in methods or uses as described above. Typically the rotavirus vaccine used is the ECACC deposit 99081301, or is derived from that deposit.

In a specific aspect the vaccine induces a cross-protective immune response or cross-protection against gastro-enteritis in a vaccinated individual compared to the unvaccinated individual (from the placebo group). Suitably the vaccine provides cross-protection against rotavirus infection symptoms such as diarrhea or gastro-enteritis. For example gastro-enteritis may be defined as diarrhea characterised by three or more, watery or looser than normal, stools within a day, or forceful vomiting along with the detection of rotavirus in the examined stool specimen.

As will be understood by the skilled artisan, disease severity and efficacy of the vaccination to induce a protective immune response in a vaccinated individual or a vaccinated population may be assessed by several means. By protective immune response is meant an immune response which leads to a reduction of the severity of clinical symptoms associated with rotavirus infection or that leads to reduced susceptibility to rotavirus infection. Disease severity in an unvaccinated or a vaccinated individual may be graded according to published scoring systems such as the 20-point Vesikari scale or a slightly amended version of said method (Ruuska T et al. Scand. J. Infect. Dis. 1990, 22, 259-267), or according to any other suitable system reporting and grading specific symptoms of the rotavirus infection (such as the methodoly reported in Clark HF, Borian EF, Bell LM. Protective effect of WC3 vaccine against rotavirus diarrhea in infants during a predominantly serotype 1 rotavirus season. *J Infect Dis.* 1988:570-86). According to the Vesikari method, severe RVGE is usually defined as a score $\geq$11.

Protection may be assessed at the level of a population or a group by vaccine efficacy (VE). Vaccine efficacy is calculated using the following formula:

VE (%) = 1 – RR = 1 – (ARV/ARU), with

RR = relative risk = ARV/ARU

ARU = disease attack rate in unvaccinated population (estimated from the placebo group) = number of subjects reporting at least one RV GE episode / total number of subjects in the control group.

ARV = disease attack rate in vaccinated group = number of subjects reporting at least one RV GE episode / total number of subjects in the HRV vaccine group.

Accordingly, in one aspect of the present invention, there is provided a method or use as detailed above wherein the composition comprising an attenuated rotavirus strain of a GxPy type induces a cross-protective immune response and/or protection against rotavirus-induced gastro-enteritis, suitably against severe rotavirus-induced gastroenteritis, caused by infection of a rotavirus strain which is neither a Gx nor a Py type. In a specific embodiment said protective immune response is capable of reducing the severity of the disease or eliminate rotavirus induced disease as measured according to any suitable scoring system.

In still another embodiment, there is provided a method or use of the composition according to the invention, to reduce the severity of the disease, e.g. gastroenteritis, or to eliminate rotavirus induced disease, said disease severity or disease being recorded according to any suitable scoring system as taught above.

In a specific embodiment, said composition is up to 60% protective, suitably up to 81% protective, in a population of vaccinated individuals, against diarrhoea caused by infection of a rotavirus of a different type to that of the attenuated rotavirus present in the composition. In another specific embodiment, said composition is at least 40% protective, suitably at least 45% protective, suitably at least 50% protective, suitably at least 60% protective, in a population of vaccinated individuals, against diarrhoea caused by a rotavirus strain which is neither a Gx nor a Py type. In a specific aspect said composition is between 40% and 80% protective, suitably between 50% and 70% protective against diarrhoea caused by a rotavirus strain which is neither a Gx nor a Py

type. In a specific aspect, said composition comprises a G1P[8] rotavirus strain which affords the level of protection as mentioned above against gastro-enteritis caused by infection of rotavirus strains of G2P[4] type.

Suitably the protection rate against diarrhoea and/or gastro-enteritis and/or severe gastro-enteritis achieved in a population of vaccinated individuals infected by a rotavirus strain which is neither a Gx nor a Py type, is between 10 to 90%, suitably between 20 to 80%, suitably between 40% and 80%, suitably between 45% and 75% protective. Typically the level of protection against severe gastro-enteritis is at least 40%, suitably at least 50%.

In a specific aspect, said composition comprises a G1P[8] rotavirus strain which is between 40% and 80% protective, suitably between 45% and 75% protective, in a population of vaccinated individuals against severe gastro-enteritis, as measured according to the Vesikari score, caused by infection of rotaviruses of with a G2P[4] serotype.

Suitably the vaccine is used in a 2 dose or a 3 dose regime.

The rotavirus vaccine used to give cross protection has the following suitable features.

In one aspect, the rotavirus of the composition for use according to the invention has a VP4 gene comprising a nucleotide sequence comprising at least one of the following: an adenine base (A) at position 788, an adenine base (A) at position 802 and a thymine base (T) at position 501 from the start codon.

In a further aspect the rotavirus of the composition for use according to the invention has a VP7 gene comprising a nucleotide sequence comprising at least one of the following: a thymine (T) at position 605, an adenine (A) at position 897, or a guanine (G) at position 897 from the start codon. Suitably at position 897 there is an adenine (A).

In a specific aspect the rotavirus of the composition for use according to the invention has an adenine (A) at positions 788 and 802 and a thymine (T) at position 501 from the start codon in the VP4 gene sequence.

In another specific aspect the rotavirus of the composition for use according to the invention has a thymine (T) at position 605 and an adenine/guanine (A/G) at position 897 from the start codon in the VP7 sequence. Most suitably in the VP7 sequence there is an adenine (A) at position 897.

In a particularly suitable aspect the rotavirus of the composition for use according to the invention has an adenine (A) at positions 788 and 802 and a thymine (T) at position 501 from the start codon in the VP4 gene sequence, and a thymine (T) at position 605 and an adenine/guanine (A/G) at position 897 from the start codon in the VP7 sequence. Suitably in the VP7 sequence there is an adenine (A) at position 897.

In another aspect the rotavirus of the composition for use according to the invention comprises a nucleotide sequence encoding a VP4 protein wherein the nucleotide sequence is as shown in Figure 1A (SEQ ID NO:1) or Figure 1B (SEQ ID NO:2), and/or a nucleotide sequence encoding a VP7 protein wherein the nucleotide sequence is as shown in Figure 2A (SEQ ID NO:3) or Figure 2B (SEQ ID NO:4). In an alternative embodiment the rotavirus of the composition for use according to the invention comprises a VP4 protein as set forth is Figure 3 (SEQ ID NO:5), and/or a VP7 protein as set forth is Figure 4 (SEQ ID NO:6). In another embodiment, said rotavirus population for use according to the invention additionally comprises an NSP4 protein as set forth in Figure 5 (SEQ ID NO:7), or encoded by the nucleotide sequence as set forth in Figure 6 (SEQ ID NO:8), and/or a VP6 protein as set forth in Figure 7 (SEQ ID NO:9), or encoded by the nucleotide sequence as set forth in Figure 8 (SEQ ID NO:10).

Suitable rotavirus populations for use in the present invention may be obtained by a method comprising:
passaging a rotavirus preparation on a suitable cell type;
optionally selecting homogeneous culture using the steps of either:
a) limit dilution; or
b) Individual plaque isolation; and
checking for the presence of a substantially single variant by carrying out a sequence determination of an appropriate region of the VP4 and/or VP7 gene sequence.

Suitably the rotavirus population is derived from the P43 (RIX4414), P33 or P26 strains as described above.

The sequence determination may suitably be carried out by a quantitative or semi-quantitative hybridisation technique such as slot blot hybridisation or plaque hybridisation.

The resulting cloned virus population resulting from the method according to the invention may be amplified by further passaging on a suitable cell line.

Suitable cell types for passaging the rotavirus population in the above method include African green monkey kidney (AGMK) cells, which may be established cell lines or primary AGMK cells. Suitable AGMK cell lines include for example Vero (ATCC CCL-81), DBS-FRhL-2 (ATCC CL-160), BSC-1 (ECACC 85011422) and CV-1 (ATCC CCL-70). Also suitable are MA-104 (rhesus monkey) and MRC-5 (human – ATCC CCL-171) cell lines. Vero cells are particularly Suitable for amplification purposes. Passaging on Vero cells gives a high virus yield.

Techniques for checking whether there is a single variant in a virus population resulting from the method, and for determining the nature of that single variant involve standard sequencing or hybridisation procedures known in the art and are described hereinbelow.

In a specific aspect the method of the invention is carried out using an appropriate rotavirus, particularly rotavirus having the characteristics of the 89-12 strain or of a passaged derivative thereof.

A particularly suitable single variant population is P43, which was obtained from P33 (an isolated human rotavirus passages 33 times in culture on appropriate cell types) by a series of end dilution cloning steps followed by passaging the cloned material on Vero cells for amplification.

A P43 population was deposited at the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury,

Wiltshire, SP4 0JG, United Kingdom on 13 August 1999 under the deposition number 99081301, under the terms of the Budapest Treaty, and is disclosed in WO 01/12797.

Although this indicated public availability is the simplest method of obtaining the human rotavirus P43, similar and functionally substantially identical rotaviruses may be produced by these or other methods in view of the teachings of this invention. Such functionally substantially identical rotaviruses are considered to be biologically equivalent to the human rotavirus P43 of this invention and therefore are within the general scope of the present invention. It will therefore be understood that the invention encompasses rotavirus populations having the characteristics of the P43 variant as described herein.

It will also be understood that the invention encompasses materials derived from the deposited P43 ECACC 99081301 by subjecting it to further processing such as by propagating it by further passaging, cloning, or other procedures using the live virus or by modifying P43 in any way including by genetic engineering techniques or reassortant techniques. Such steps and techniques are well known in the art.

Materials derived from the deposited P43 which are covered by the invention include protein and genetic material. Of particular interest are reassortant rotaviruses which comprise at least one antigen or at least one segment of P43, for example reassortants which comprise a virulent strain of rotavirus in which one or part of one of the 11 genome segments has been replaced by the genome segment or part thereof of P43. Specifically, a rotavirus reassortant in which the segment or partial segment coding for NSP4 is a P43 segment or partial segment, may have useful properties. Reassortant rotaviruses and techniques for preparing them are well known (Foster, R. H. and Wagstaff, A. J. Tetravalent Rotavirus Vaccine, a review. ADIS drug evaluation, BioDrugs, Gev, 9 (2), 155-178, 1998).

Materials of particular interest are progeny of P43 and immunologically active derivatives of P43. Immunologically active derivatives means materials obtained from or with the P43 virus, particularly antigens of the virus, which are capable of eliciting an immune response that is reactive against Rotavirus when injected into a host animal.

In adapting the rotavirus to an appropriate cell line, for example Vero cells, it may be necessary to treat the virus so as to get rid of any potential contaminant such as any adventitious agents that may be present and which would otherwise cause contamination. In the case of ether-sensitive adventitious viruses, this may be done by ether treatment as described hereinbelow. The present invention also relates to inclusion of such ether treatment as an optional step in the overall procedure for obtaining an attenuated live rotavirus or vaccine formulated therewith.

The cross protective rotavirus strain of the present invention may be combined with other rotavirus strains to provide additional protection or cross-protection against rotavirus infection or disease.

The present invention also provides a live attenuated rotavirus vaccine capable of providing cross protection, as defined herein above, admixed with a suitable adjuvant or a pharmaceutical carrier.

In one embodiment, the rotavirus vaccine for use according to the invention is a monovalent rotavirus vaccine containing a single rotavirus strain such as the G1P[8] strain.

The present invention is particularly advantageous in providing a live rotavirus vaccine in which the live attenuated rotavirus is a human rotavirus and does not cause intussusception.

Suitable pharmaceutical carriers for use with the attenuated rotavirus strain according to the invention include those known in the art as being suitable for oral administration, especially to infants. Such carriers include and are not limited to carbohydrates, polyalcohols, amino acids, aluminium hydroxide, magnesium hydroxide, hydroxyapatite, talc, titanium oxide, iron hydroxide, magnesium stearate, carboxymethylcellulose, hydroxypropylmethylcellulose, microcrystalline cellulose, gelatin, vegetal peptone, xanthane, caraghenane, arabic gum, ß-cyclodextrin.

The invention also provides a process for preparing a rotavirus vaccine, for example by freeze drying the virus in the presence of suitable stabilisers or admixing the virus according to the invention with a suitable adjuvant or pharmaceutical carrier.

It may also be advantageous to formulate the virus of the invention in lipid-based vehicles such as virosomes or liposomes, in oil in water emulsions or with carrier particles. Alternatively or in addition immunostimulants such as those known in the art for oral vaccines may be included in the formulation. Such immunostimulants include bacterial toxins, particularly cholera toxin (CT) in the form of the holotoxin (entire molecule) or the B chain only (CTB) and the heat labile enterotoxin of *E. coli* (LT). Mutated LTs (mLTs) which are less likely to convert to their active form than the native LT are described in WO 96/06627, WO 93/13202 and US 5,182,109.

Further immunostimulants which may advantageously be included are saponin derivatives such as QS21 and monophosphoryl lipid A, in particular 3-de-O-acylated monophosphoryl lipid A (3D-MPL). Purified saponins as oral adjuvants are described in WO 98/56415. Saponins and monophosphoryl lipid A may be employed separately or in combination (e.g. WO 94/00153) and may be formulated in adjuvant systems together with other agents. 3D-MPL is a well-known adjuvant manufactured by Ribi Immunochem, Montana and its manufacture is described in GB 2122204.

A general discussion of vehicles and adjuvants for oral immunisation can be found in Vaccine Design, The Subunit and Adjuvant Approach, edited by Powell and Newman, Plenum Press, New York, 1995.

The invention also provides a method for vaccinating human subjects, especially infants, by administering to a subject in need thereof an effective amount of a vaccine composition according to the invention. Suitably the live attenuated vaccine is administered by oral administration.

In a specific aspect the attenuated rotavirus strain according to the invention is formulated with an antacid to minimise inactivation of the vaccine by acid in the stomach. Suitable antacid components include inorganic antacids for example aluminium hydroxide $Al(OH)_3$ and magnesium hydroxide $Mg(OH)_2$. Commercially available

antacids which are suitable for use in the invention include Mylanta (trade mark) which contains aluminium hydroxide and magnesium hydroxide. These are insoluble in water and are given in suspension.

Aluminium hydroxide is a particularly suitable component of a vaccine composition according to the invention as it can provide not only an antacid effect but also an adjuvantation effect.

Also suitable for use as antacids in the vaccine of the invention are organic antacids such as organic acid carboxylate salts. A suitable antacid in the vaccine composition of the invention contains an organic acid carboxylate salt, specifically a salt of citric acid such as sodium citrate or potassium citrate.

A particularly suitable antacid that may be used in the vaccine composition of the present invention is the insoluble inorganic salt, calcium carbonate ($CaCO_3$). The calcium carbonate is able to associate with the rotavirus and the rotavirus activity is maintained during the association with the calcium carbonate.

To prevent sedimentation of calcium carbonate during the filling step, viscous agents are suitably present in the formulation.

Possible viscous agents that may be used include pseudoplastic excipients. A pseudoplastic solution is defined as a solution having higher viscosity on standing compared to its viscosity under agitation. Excipients of this type are natural polymers such as arabic gum, adragante gum, agar-agar, alginates, pectines or semi-synthetic polymers for example: carboxymethylcellulose (Tyloses C®), methylcellulose (Methocels A®, Viscotrans MC®, Tylose MH® and MB®), hydroxypropylcellulose (Klucels®), and hydroxypropylmethylcellulose (Methocels E® and K®, Viscontrans MPHC®). In general those pseudoplastic excipients are used together with thixotropic agents. Alternative viscous agents that may be used are pseudoplastic excipients with low flowing capacity. Those polymers, at a sufficient concentration, give rise to a structural fluid arrangement resulting in a high viscosity solution having low flowing capacity on standing. A certain quantity of energy needs to be given to the system to allow flowing and transfer.

External energies (agitation) are needed to destroy temporarily the structural fluid arrangement in order to obtain a fluid solution.
Examples of such polymers are Carbopols® and xanthane gum.

Thixotropic excipents become a gel structure on standing whilst under agitation they form a fluid solution. Examples of thixotropic excipients are: Veegum ®(Magnesium-aluminium silicate) and Avicel RC® (about 89% microcrystalline cellulose and 11% Carboxymethylcellulose Na).

The vaccine composition of the present invention suitably comprises a viscous agent selected from xanthane gum or starch.

Thus the vaccine composition of the present invention is typically formulated with a combination of calcium carbonate and xanthane gum.

Other components of a composition used in the invention suitably include sugars for example sucrose and/or lactose.

The vaccine composition according to the invention may contain additional components including for example flavourings (particularly for an oral vaccine) and bacteriostatic agents.

Different presentations of the vaccine composition according to the invention are envisaged.

In one suitable embodiment, the vaccine is administered as a liquid formulation. Suitably the liquid formulation is reconstituted prior to administration from at least the following two components:

i) virus component
ii) liquid component.

In this embodiment, the virus component and the liquid component are normally present in separate containers, which may conveniently be separate compartments of a single

vessel, or separate vessels which can be connected in such a way that the final vaccine composition is reconstituted without exposing it to the air.

Prior to reconstitution, the virus may be in a dry form or a liquid form. Suitably the virus component is lyophilised. Lyophilised virus is more stable than virus in an aqueous solution. The lyophilised virus may be suitably reconstituted using a liquid antacid composition to produce a liquid vaccine formulation. Alternatively the lyophilised virus may be reconstituted with water or aqueous solution, in which case the lyophilised virus composition suitably contains an antacid component.

Suitably, the vaccine formulation comprises a virus component formulated with calcium carbonate and xanthane gum in one compartment or vessel and this is reconstituted with water or aqueous solution present in the second compartment or vessel.

In another embodiment, the vaccine composition is a solid formulation, suitably a lyophilised cake which is suitable for immediate dissolution when placed in the mouth. Lyophilised formulations may conveniently be provided in the form of tablets in a pharmaceutical blister pack.

In another aspect the invention provides a rotavirus vaccine in the form of a quick dissolving tablet for oral administration.

In another aspect the invention provides a composition comprising a live attenuated rotavirus strain, in particular a human rotavirus strain, wherein the composition is a lyophilised solid capable of immediate dissolution when placed in the mouth.

Suitably the quick dissolving tablet according to the invention dissolves in the mouth of the subject sufficiently quickly to prevent swallowing of the undissolved tablet. This approach is particularly advantageous for paediatric rotavirus vaccines.

Suitably the virus is a live attenuated human rotavirus which is formulated with an inorganic antacid such as calcium carbonate and a viscous agent such as xanthane gum.

A further aspect of the present invention is to provide a lyophilised formulation wherein the virus component is any rotavirus strain which is formulated with calcium carbonate and xanthane gum.

Vaccines of the invention may be formulated and administered by known techniques, using a suitable amount of live virus to provide effective protection against rotavirus infection without significant adverse side effects in typical vaccinees. A suitable amount of live virus will normally be between 10^4 and 10^7 focus forming units (ffu) per dose. A typical dose of vaccine may comprise $10^5 - 10^6$ ffu per dose and may be given in several doses over a period of time, for example in two doses given with a two-month interval. Benefits may however be obtained by having more than 2 doses, for example a 3 or 4 dose regimen, particularly in developing countries. The interval between doses may be more or less than two months long. An optimal amount of live virus for a single dose or for a multiple dose regimen, and optimal timing for the doses, can be ascertained by standard studies involving observation of antibody titres and other responses in subjects.

The vaccine of the invention may also comprise other suitable live viruses for protection against other diseases, for example poliovirus. Alternatively other suitable live virus vaccines for oral administration may be given in a separate dose but on the same occasion as the rotavirus vaccine composition according to the invention.

Sera from twelve 4 to 6 month old infants vaccinated with the P33 material as described in the Vaccine (1998) paper were tested for neutralization of P33, P38, P43 and 89-12C2.

The range of neutralization titers of all the tested sera is similar for P33, P38 and P43. The statistical analysis shows no significant difference in the overall neutralization titers against all three viruses. This suggests that the conformational and non-conformational neutralization epitopes of P33, P38 and P43 are equally well recognized by the anti-P33 sera of P33 vaccinated infants. This observation indirectly suggests that the neutralization epitopes revealed in this in vitro assay were not altered between P33, P38 and P43.

The range of neutralization titers of P89-12C2 however significantly differs from P33, P38 and P43. This observation suggests that the conformational and non-conformational neutralization epitopes of P33, P38 and P43 are not equally well recognized by the anti-P33 sera of P33 vaccinated infants. This observation indirectly suggests that the neutralization epitopes revealed in this in vitro assay were altered between 89-12 C2 and P33, P38 and P43.

Particularly suitable embodiments of the present invention include:

1. The use of an attenuated rotavirus strain from a P type in the manufacture of a vaccine composition for the induction of an immune response against a rotavirus of a different P type to that of said vaccine composition.

2. The use of an attenuated rotavirus strain from a P[8] type in the manufacture of a vaccine composition for the induction of an immune response against a rotavirus which is not P[8].

3. The use of an attenuated rotavirus strain from a G1P[8] type in the manufacture of a vaccine composition for the induction of an immune response against a rotavirus which is not G1P[8].

4. The use according to 1 to 3 wherein an immune response is additionally induced against rotavirus infection by a G1P[8] type.

5. The use according to 1 to 4 wherein the immune response is induced against two or more rotavirus serotypes, these serotypes being defined by reference to the G or P types.

6. The use according to 1 to 5 wherein the serotype of the vaccine strain is a G1 serotype and the non-G1 serotype is selected from the list consisting of: G2, G3, G4, G5, G6, G7, G8, G10, G11, G12, G13 and G14.

7. The use according to 6 wherein an immune response is induced against both the G1 type and the G2 type.

8. The use according to any of 1 to 5 wherein the type of the vaccine strain is a P[8] type and the non- P[8] type is selected from the list consisting of: P[1], P[2], P[3], P[4], P[5], P[6], P[7], P[9] and P[11] types.

9. The use according to 8 wherein an immune response is induced against both the P[8] and the P[4] types.

10. The use according to any of 1 to 9 wherein the composition comprises a rotavirus having a VP4 gene comprising, in the nucleotide sequence, at least one of the following: an adenine base (A) at position 788, an adenine base (A) at position 802 and a thymine base (T) at position 501 from the start codon.

11. The use according to 10 wherein the VP4 gene comprises a nucleotide sequence comprising an adenine base (A) at positions 788 and 802 and a thymine base (T) at position 501 from the start codon.

12. The use according to 11 wherein the composition comprises a rotavirus having a VP7 gene comprising, in the nucleotide sequence, at least one of the following: a thymine (T) at position 605, an adenine (A) at position 897 and a guanine (G) at position 897 from the start codon.

13. The use according to 12 wherein the VP7 gene comprises a nucleotide sequence comprising a thymine (T) at position 605 and an adenine (A) or a guanine (G) at position 897 from the start codon.

14. The use according to any of 1 to 13, wherein the composition comprises a rotavirus having a VP4 gene comprising, in the nucleotide sequence, an adenine (A) at positions 788 and 802 and a thymine (T) at position 501 from the start codon; and wherein the VP7 gene comprises, in the nucleotide sequence, a thymine (T) at position 605 and an adenine (A) at position 897 from the start codon.

15. The use according to any of 1 to 14 wherein the composition is capable of reducing or protecting against gastro-enteritis and/or diarrhea caused by infection by a

rotavirus of a different type defined by reference to either the G and/or the P type of the attenuated rotavirus present in the composition.

16. The use according to 15 wherein the composition is at least 40% protective in a population of vaccinated individuals against severe gastro-enteritis caused by infection of rotaviruses of at least two strains defined by reference to either the G and/or the P type, these types being different to the G1P[8] type of the attenuated rotavirus present in the composition.

17. The use according to 16 wherein the severe gastro-enteritis is caused by infection of a rotavirus of at least three, at least four, non-G1 serotypes.

18. The use according to 17 wherein the non-G1 serotypes are any of G2, G3, G4 and G9 serotypes.

19. The use according to 18 wherein the severe gastro-enteritis is caused by infection of a rotavirus of at least two non-P[8] types.

20. The use according to 9 wherein the severe gastro-enteritis is caused by infection of a rotavirus of a P[4] type.

21. The use according to any of 1 to 20 wherein the rotavirus strain is ECACC deposit 99081301, or is obtainable or derivable from ECACC deposit 99081301.

23. The use according to any of 1 to 20 wherein the vaccine is used in a 2-dose regime.

In another aspect, the invention also relates to a method of inducing an immune response against rotavirus infection from a rotavirus strain, the method comprising administering to a subject a composition comprising an attenuated rotavirus vaccine from a different strain. Specifically the invention relates to a method for inducing an immune response against rotavirus from one P type and/or for preventing disease associated with rotavirus infection from one P type, said method comprising administering to a patient in need thereof an attenuated rotavirus population from a different P type.

In a specific aspect of the invention there is provided a method of inducing an immune response to P[8] rotavirus type and at least one of the non-P[8] types selected from the group consisting of: P[1], P[2], P[3], P[4], P[5], P[6], P[7], P[9], P[11], P[12] , P[14] and P[19] types, suitably to the P[4] rotavirus type, the method comprising administering to a subject a composition comprising a rotavirus P[8] type vaccine.

In another aspect of the invention there is provided i) an isolated non-structural protein 4 (NSP4) protein sequence as set forth in Figure 5 (SEQ ID NO:7) or immunogenic fragment thereof; ii) an isolated polynucleotide sequence which comprises a nucleic acid sequence encoding said NSP4 polypeptide, or immunogenic fragment thereof; iii) an isolated polynucleotide sequence which comprises a nucleic acid sequence as set forth in Figure 6 (SEQ ID NO:8).

In still another aspect of the invention there is provided i) an isolated rotavirus protein 6 (VP6) protein sequence as set forth in Figure 7 (SEQ ID NO:9) or immunogenic fragment thereof; ii) an isolated polynucleotide sequence which comprises a nucleic acid sequence encoding said VP6 polypeptide, or immunogenic fragment thereof; iii) an isolated polynucleotide sequence which comprises a nucleic acid sequence as set forth in Figure 8 (SEQ ID NO:10).

Immunogenic fragments may be defined in the context of this invention as fragments that when administered at an effective dose (either alone or as a hapten bound to a carrier) elicit a protective immune response against rotavirus infection.

The following, non-limiting, examples illustrate the invention.

EXAMPLES

EXAMPLE 1: Demonstration that strain 89-12 at passage 26 (P26) is a mixture of variants

Sequencing of VP4 and VP7 genes from different passage lots

Sequencing of VP4 and VP7 genes from passage P26 (primary AGMK cells), passage P33 (established (as opposed to primary) AGMK cell line), passage P41 and passage P43 was performed. Total RNA extraction was reverse transcribed and amplified through PCR in one tube/one step.

Primers Rota 5bis and Rota 29bis amplified the entire VP4 gene and primers Rota 1 and Rota 2bis amplified the entire VP7 gene. The PCR material has been sequenced using different primers (see Table 1).

The passage P26 sequence differed from the passage P33 sequence by 3 bases (at positions 501, 788 and 802 bp from the start codon) in VP4 and by three bases in VP7 (108, 605 and 897 bp from the start codon).

The passage P26 sequence scans of VP4 and VP7 show at mutated positions the presence of the passage P33 sequence as a background. Thus it can be seen that passage P26 is a mixture of at least 2 variants.

The passage P33 sequence scans seem homogenous in VP4 and heterogeneous for VP7 (see Table 2).

Passage P38 (derived from passage 33) was passaged 5 times on Vero cells and displayed the same set of VP4 and VP7 sequences as passage P33 (AGMK cell line). Thus there was no major change in populations between P33 and P38.

TABLE 1: Oligonucleotides used for RT-PCR and sequencing

	name	sequence	position
VP7	Rota 1	GGC TTT AAA AGA GAG AAT TTC CGT CTG G (SEQ ID NO:11)	-49 to -22
	Rota 1bis	GGT TAG CTC CTT TTA ATG TAT GGT A (SEQ ID NO:12)	-16 to 10
	Rota 2bis	GGT CAC ATC GAA CAA TTC TAA TCT AAG (SEQ ID NO:13)	1014-988
	Rota 7	CAA GTA CTC AAA TCA ATG ATG G (SEQ ID NO:14)	266-287
	Rota 12	TGT TGA TTT TTC TGT CGA TCC AC (SEQ ID NO:15)	372-394
	Rota 46	GGT TGC TGA GAA TGA GAA ATT AGC TAT AGT GG (SEQ ID NO:16)	651-682
	Rota 18	CCA CTA TAG CTA ATT TCT CAT TCT CAG CAA CC (SEQ ID NO:17)	682-651
VP4	Rota 5	TGG CTT CGC CAT TTT ATA GAC A (SEQ ID NO:18)	2-23
	Rota 6	ATT TCG GAC CAT TTA TAA CC (SEQ ID NO:19)	878-859
	Rota 5bis	TGG CTT CAC TCA TTT ATA GAC A (SEQ ID NO:20)	2-23
	Rota 6bis	ATT TCA GAC CAT TTA TAA CCT AG (SEQ ID NO:21)	878-856
	Rota 25	GGA GTA GTA TAT GAA AGT ACA AAT AAT AG (SEQ ID NO:22)	268-296
	Rota 26	CTA TTA TTT GTA CTT TCA TAT ACT ACT CC (SEQ ID NO:23)	296-268
	Rota 27bis	TCG ATA CAG TAT AAG AGA GCA CAA G (SEQ ID NO:24)	721-745
	Rota 28	TTC ATT AAC TTG TGC TCT CTT ATA CTG (SEQ ID NO:25)	
	Rota 31	GTA TAT GTA GAC TAT TGG GAT G (SEQ ID NO:26)	753-727
	Rota 32	CAT CCC AAT AGT CTA CAT ATA C (SEQ ID NO:27)	1048-1070
	Rota 45	TGT AAC TCC GGC AAA ATG CAA CG (SEQ ID NO:28)	1070-1048
	Rota 53	CGT TGC ATT TTG CCG GAG TTA CA (SEQ ID NO:29)	1205-1227
	Rota 54	GTA AGA CAA GAT TTA GAG CGC CA (SEQ ID NO:30)	1227-1205
	Rota 55	TGG CGC TCT AAA TCT TGT CTT AC (SEQ ID NO:31)	1465-1487
	Rota 40	CTT GAT GCT GAT GAA GCA GCA TCT G (SEQ ID NO:32)	1487-1465
	Rota 39	CAG ATG CTG CTT CAT CAG CAT CAA G (SEQ ID NO:33)	1703-1727
	Rota 33	CGA TCA TAT CGA ATA TTA AAG GAT G (SEQ ID NO:34)	1727-1703
	Rota 34	CAT CCT TTA ATA TTC GAT ATG ATC G (SEQ ID NO:35)	2008-2032
	Rota 29bis	AGC GTT CAC ACA ATT TAC ATT GTA G (SEQ ID NO:36)	2032-2008
			2335-2311

TABLE 2: oligonucleotides used in hybridization

	name	sequence	position
VP7	Rota 41	AGT ATT TTA TAC TAT **A**GT AGA TTA TAT TAA TC (SEQ ID NO:37)	882-913
	Rota 42	AGT ATT TTA TAC TAT **G**GT AGA TTA TAT TAA TC (SEQ ID NO:38)	882-913
VP4	Rota 15	ATC CC**C** ATT ATA CTG CAT T**C**C TTT C (SEQ ID NO:39)	807-783
	Rota 16	ATC CC**T** ATT ATA CTG CAT T**T**C TTT C (SEQ ID NO:40)	807-783
	Rota 35	ATC CC**C** ATT ATA CTG CAT T**T**C TTT C (SEQ ID NO:41)	807-783
	Rota 36	ATC CC**T** ATT ATA CTG CAT T**C**C TTT C (SEQ ID NO:42)	807-783

The bases shown in bold type in Table 2 are the sites of specific sequence variation in VP4 and VP7.

TABLE 3: sequence variation of VP4 and VP7 genes

Table 3.1

	VP4			VP7		
	501 bp 167 aa	788 bp 263 aa	802 bp 268 aa	108 bp 36 aa	605 bp 202 aa	897 bp 299 aa
P26 (AGMK)	A	G / A	G / A	A	C / T	A
P33 (AGMK)	T	A	A	G / A	T / C	A / G
P38 (VERO)	T	A	A	A / G	T	G / A
P43 (VERO)	T	A	A	A	T	A

N.B. In a second clone from the 3 clones which were developed to the level of production lot, the VP7 897 bp position nucleotide is G, rather than A as in the P43 selected clone. This results in a methionine in place of an isoleucine in the amino acid sequence. Variants corresponding to both the selected P43 clone and the clone in which there is a G in VP7 at 897 bp from the start codon, were excreted in the stools of infants who had been vaccinated with the P33 material.

In Table 3.1, where there are two alternative bases at a particular position, the first of the two represents the base which appears in a major population and the second is the base which appears in a minor population. Major and minor variant populations are judged by the strength of the signal in sequencing.

Table 3.2

	VP4			VP7		
	501 bp 167 aa	788 bp 263 aa	802 bp 268 aa	108 bp 36 aa	605 bp 202 aa	897 bp 299 aa
P26 (AGMK)	Leu	Gly/Glu	Gly/Arg	Arg	Thr/Met	Ile
P33 (AGMK)	Phe	Glu	Arg	Arg/Arg	Met/Thr	Ile/Met
P38 (VERO)	Phe	Glu	Arg	Arg/Arg	Met	Met/Ile
P43 (VERO)	Phe	Glu	Arg	Arg	Met	Ile

Table 3.2 shows the amino acid changes resulting from the nucleotide differences between the variants.

TABLE 4

	VP4 (788 -802 positions)				VP7 (897 position)	
	G-G	A-A	A-G	G-A	A	G
Probes	Rota 15	Rota 16	Rota 35	Rota 36	Rota 41	Rota 42
Passages						
P26	-	+	+	+	nd	nd
P33	-	+	-	-	+ +	+
P38	-	+	-	-	+	++
P43	-	+	-	-	+	-

Slot blot hybridization

The change in populations between passages P26 to P33 on AGMK cells has been further confirmed by slot blot hybridization. The VP4 and the VP7 gene fragments generated by RT/PCR were hybridized with oligonucleotide probes specific for each variant (see Table 3.1 and 3.2). In contrast to P26 which hybridized with Rota 16, Rota 35 and Rota 36 and not with Rota 15, the VP4 PCR fragment of the P33 material, at positions 788 and 802 hybridized only with Rota 16 and not with either Rota 15 or Rota 35 or Rota 36. These results established the presence of at least 3 variants in P26 (see Table 4).

For the VP7 PCR fragment of the P33 material, position 897 hybridized with Rota 41 and Rota 42. These results established the presence of at least two variants in the P33 material.

EXAMPLE 2: Isolation and characterization of the P43 clone

To isolate P33 components as a homogeneous virus population, three end-point dilutions of P33/AGMK on Vero cells were performed and the resulting virus was used to infect Vero cells.

Positive wells were selected using two criteria: growth demonstrated by the largest number of foci detected in the wells and the most isolated positive wells on the plates, as

is done classically. After 3 end dilution passages in 96 well microtiter plates, 10 positive wells were amplified successively on Vero cells and evaluated for their yield.
Based on yield, three clones were developed to passage level of production lot.
Immunorecognition by polyclonal antibodies was shown to be similar both between the three clones and between the clones and P33. Homogeneity of the clones was assessed by slot blot hybridization. The final selection of a single clone was based on yield and sequence.
The selected clone was amplified by successive passages on Vero cells to generate a Master seed, a Working seed and finally production lots.
The selected clone was genetically characterized at different passage levels by sequencing of VP4 and VP7 (identity) and by specific slot blot hybridization of the VP4 and VP7 (homogeneity) of the PCR amplified materials. The sequence of the VP4 and VP7 genes of the P43 material are given in Figures 1 and 2 respectively and are identical to P41.

Homogeneity of the selected clone was assessed by a selective hybridization using oligonucleotide probes discriminating nucleotide changes in VP4 and/or VP7 regions for each variant identified during sequencing of P26/primary AGMK (see Table 4).

The VP4 fragment hybridized with Rota 16 and not with Rota 15, Rota 35 or Rota 36.
The VP7 fragment hybridized with Rota 41 and not with Rota 42.

These results confirmed that P43 is a homogeneous population.

EXAMPLE 3: Removal of potential adventitious virus

Ether was added to P33 (AGMK grown) to a final concentration of 20% for 1 hr. Ether was then bubbled out with N_2 for 35 min. No impact on the titre of P33 seed was observed.

EXAMPLE 4: Formulation of a live attenuated vaccine

The production lots described above are formulated for oral adminstration to infants by the following method.

1. Lyophilised virus

Standard techniques are used for preparing virus doses. Frozen purified viral bulk is thawed and diluted with appropriate medium composition, in this case Dulbecco's modified eagle Medium, up to a desired standard viral concentration, in this case $10^{6.2}$ ffu/ml. The diluted virus is then further diluted with lyophilisation stabiliser (sucrose 4%, dextran 8%, sorbitol 6%, amino-acid 4%) up to the target viral titre, in this case $10^{5.6}$ ffu/dose. 0.5 ml aliquots of stabilised virus composition are aseptically transferred to 3 ml vials. Each vial is then partially closed with a rubber stopper, the sample is freeze dried under a vacuum, the vial is then fully closed and an aluminium cap is crimped in place around the vial to keep the stopper in place.

For use, the virus is reconstituted using one of the following antacid reconstituents:

(a) Citrate reconstituent

Sodium citrate is dissolved in water, sterilized by filtration and aseptically transferred into reconstituent containers in 1.5 ml amounts at a concentration of 544 mg $Na_3Citrate.2H_2O$ per 1.5ml dose. The reconstituent containers may be for example 3 ml vials, or 4 ml vials, or 2 ml syringes, or soft plastic squeezable capsules for oral administration. As an alternative to maintaining sterile components under sterile conditions, the final container can be autoclaved.

(b) $Al(OH)_3$ reconstituent

An aseptic aluminium hydroxide suspension (Mylanta – trademark) is aseptically diluted in sterile water, aseptically transferred to reconstituent containers (for example 2 ml syringes, or soft plastic squeezable capsules) in 2 ml amounts each containing 48 mg $Al(OH)_3$. An alternative to using sterile components under sterile conditions is to γ irradiate the aluminium hydroxide suspension (preferably at a diluted stage).

Standard ingredients are included to prevent the suspension from settling. Such standard ingredients include for example magnesium stearate, carboxymethylcellulose, hydroxypropylmethylcellulose, microcrystalline cellulose, and silicone polymers.

Bacteriostatic agents for example butylparaben, propylparaben or other standard bacteriostatic agents used in food, and flavourings, may also be included.

2. Lyophilised virus with $Al(OH)_3$ in liquid formulation

Standard techniques are used for preparing virus doses. Frozen purified viral bulk is thawed and diluted with appropriate medium composition, in this case Dulbecco's modified eagle Medium, up to a desired standard viral concentration, in this case $10^{6.2}$ ffu/ml. Aluminium hydroxide suspension is added to reach a final quantity of 48 mg/dose and the virus composition is diluted with lyophilisation stabiliser (sucrose 4%, dextran 8%, sorbitol 6%, amino-acid 4%) up to the target viral titre, in this case $10^{5.6}$ ffu/dose. 0.5 ml aliquots of stabilised virus composition are aseptically transferred to 3 ml vials. Lyophilisation and closing of the vials is then carried out as described in part 1.

3. Lyophilised virus with $Al(OH)_3$ for blister presentation

Standard techniques are used for preparing virus doses. Frozen purified viral bulk is thawed and diluted with appropriate medium composition, in this case Dulbecco's modified eagle Medium, up to a desired standard viral concentration, in this case $10^{6.2}$ ffu/ml. Aluminium hydroxide suspension is added to reach a final quantity of 48 mg/dose and the virus composition is diluted with lyophilisation stabiliser which may be sucrose, dextran or amino-acid 4%, or gelatin, or vegetal peptone, or xanthane up to the target viral titre of $10^{5.6}$ ffu/dose. An aseptic filling operation is employed to transfer doses of 0.5 ml or preferably less to blister cavities. The composition is lyophilised, and the blister cavities are sealed by thermic sealing.

Optionally standard ingredients are included to prevent the aluminium hydroxide suspension from settling. Such standard ingredients include for example magnesium stearate, carboxymethylcellulose, hydroxypropylmethylcellulose, microcrystalline cellulose, and silicone polymers. Flavourings may also be included.

Example 5: Rotavirus viral titration for various formulations

5.1:Comparison between lactose and sucrose based formulations:

Table 5

Batch n°	Fomulation composition	Viral titer before lyophilisation	Viral titer after lyophilisation and 1 week at 37°C
98G06/01	Lactose:2%; Dextran:4%; Sorbitol:3%; AminoAcids:2%	$10^{5.22}$	$10^{4.67}$
98G06/03	Sucrose:2%; Dextran:4%; Sorbitol:3%; AminoAcids:2%	$10^{5.28}$	$10^{4.92}$

P43 rotavirus was formulated either with sucrose or with lactose as shown in the table above.
Viral titration before lyophilisation is the viral titre in the completed formulated liquid (containing sucrose dextran sorbitol aminoacids) and without the lyophilisation step.

Good results are those in which a <0.5 log decrease at the lyophilisation step and <0.5 log decrease during the "1 week at 37°C" (accelerated stability test) are achieved.
The precision of the viral titration is around + or – 0.2 log.

The results indicate that sucrose may be used instead of lactose.

5.2: Effect of arginine and replacement of sorbitol by maltitol:

Table 6

Batch n°	Fomulation composition	Viral titer at time = zero after lyophilisation	Viral titer after lyopjhilisation and 1 week at 37°C
98L16/01	Lactose:2%; Dextran:4%; Sorbitol:3%; AminoAcids:2%	$10^{4.8}$	$10^{4.8}$
98L16/02	Lactose:2%; Dextran:4%; Sorbitol:3%; AminoAcids:2%	$10^{4.8}$	$10^{4.9}$

	Arginine: 3%		
98L16/04	Lactose:2%; Dextran:4%; Maltitol:3%; AminoAcids:2% Arginine: 3%	$10^{4.7}$	10^{5}

The results demonstrate that the addition of arginine (which is known to improve the stability of the virus during lyophilisation and also provides a basic medium in order to compensate for the stomach acidity) maintains the viral titer.

Sorbitol tends to decrease the glass transition temperature of the lyophilised cake by too great a degree. This can be overcome by using maltitol instead of sorbitol as shown above and the viral titer is still maintained.

5.3: Various formulation compositions

This experiment demonstrates that a number of formulations are possible.

Table 7

Batch n°	Fomulation composition	Viral titer before lyophilisation	Viral titer after lyophilisation and 1 week at 37°
99C11/01	Sucrose:2%; Dextran:4%; Sorbitol:3%; AminoAcids:2%	$10^{5.24}$	$10^{5.07}$
99C11/02	Sucrose:2%; Dextran:4%; Maltitol:3%; AminoAcids:2%	$10^{5.09}$	$10^{4.92}$
99C11/04	Dextran:4%; Maltitol:3%; AminoAcids:2%	$10^{4.89}$	$10^{5.06}$

Batch n°	Fomulation composition	Viral titer at time = zero after lyophilisation	Viral titer after lyophilisation and 1 week at 37°
99C17/01	Sucrose:2%; Dextran:4%; Sorbitol:3%;	$10^{5.40}$	$10^{5.41}$

	AminoAcids:2%		
99C17/02	Sucrose:2%; Dextran:4%; Sorbitol:1.5%; AminoAcids:2%	$10^{5.30}$	$10^{4.93}$
99C17/03	Sucrose:2%; Dextran:4%; AminoAcids:2%	$10^{5.31}$	$10^{5.24}$
99C17/04	Sucrose:2%; Dextran:4%; Maltitol:3%; AminoAcids:2%	$10^{4.42}$	$10^{4.45}$
99C17/05	Sucrose:2%; Dextran:4%; Maltitol:1.5%; AminoAcids:2%	$10^{4.39}$	$10^{4.40}$
99C17/06	Sucrose:2%; Dextran:4%; Sorbitol:3%;	$10^{5.44}$	$10^{4.97}$
99C17/07	Sucrose:2%; Dextran:4%; Sorbitol:1.5%;	$10^{5.11}$	$10^{4.89}$

5.4: Association between Rotavirus and $Al(OH)_3$ antacid:

Table 8

Rotavirus	$Al(OH)_3$	H_2O	Contact time at room temperature	Centrifugation	Supernatant viral titer in ffu/ml	Pellets viral titer in ffu/ml
$10^{5.6}$ ffu/ml	48 mg in 0.240ml	0.76 ml	30 min	8000rpm, 10 min	$10^{3.66}$	
$10^{5.6}$ ffu/ml	0.48 mg in 0.240ml	0.76 ml	30 min	8000rpm, 10 min	$10^{4.41}$	
$10^{5.6}$ ffu/ml		1 ml	30 min	8000rpm, 10 min	$10^{5.68}$	
Rotavirus in Lyophilised Cake	12 mg in 0.120ml	1.38 0ml	30 min	8000rpm, 10 min	Below detection	$10^{4.7}$

$Al(OH)_3$ is used as an antacid. This shows that Rotavirus is associated with the insoluble inorganic salt ($Al(OH)_3$) since it centrifuged together with the $Al(OH)_3$ (decrease of viral activity in the supernatant).

5.5: Dissolution of $Al(OH)_3$ antacid by SodiumCitrate before viral titration

Table 9

Viral samples	Dissolution	Conditions	Viral titers ffu/ml
99B10/06 liquid formulation before lyophilisation; $10^{5.43}$	1.5ml $Na_3Citrate$	24h at room temperature	$10^{5.11}$
99B10/06:lyophilized $10^{5.43}$	1.5ml $Na_3Citrate$	24h at room temperature	$10^{4.53}$

When Rotavirus is associated with the $Al(OH)_3$, it is possible to lyophilise everything (including the $Al(OH)_3$). After lyophilisation, it is possible to recover the Rotavirus by dissolving $Al(OH)_3$ in SodiumCitrate. This step does not damage the Rotavirus and retains its activity after this dissolution step.

5.6: Infectivity of Rotavirus after liberation of the $Al(OH)_3$-Rotavirus association:

The mechanism of virus liberation (by dissolution of the carrier) may very well occur *in vivo*. Indeed below pH 6, aluminium hydroxide becomes completely soluble, and thus, Rotavirus will be liberated in the stomach.

$$Al(OH)_3 + 3\ H^+ \dashrightarrow Al^{+++}\ (\text{water soluble}) + 3\ H_2O$$

In the stomach, Al^{+++} ions are not absorbed (J.J. Powell, R.Jugdaohsingh and R.P.H. Thompson, *The regulation of mineral adsorption in the gastrointestinal track*, Proceedings of the Nutrition Society (1999), 58, 147-153).

In the intestine, due to the increase of pH, insoluble forms of aluminium are precipitated ($Al(OH)_3$ or $AlPO_4$), and eliminated by the natural way.

It is unknown whether the newly formed $Al(OH)_3$ (or $AlPO_4$) precipitate will be able to re-associate with free Rotavirus. This raises the question of the infectivity of the $Al(OH)_3$-Rotavirus association itself.

Liberation of Rotavirus from the $Al(OH)_3$-Rotavirus association by other mechanisms is also possible. Lysine, for example, interferes with the viral adsorption on $Al(OH)_3$. Other anions like borate, sulfate, carbonate and phosphate are known to be specifically adsorbed on aluminium hydroxide, thus, theoretically, it should be possible to displace (by competition for the adsorption site) Rotavirus from the $Al(OH)_3$-Rotavirus association.

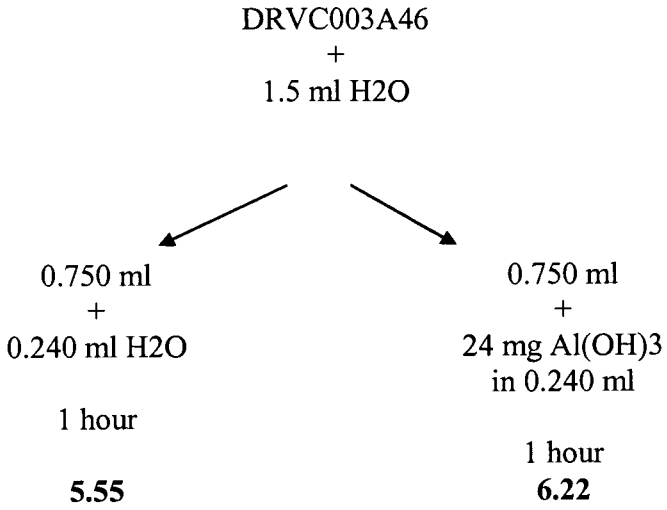
DRVC003A46
+
12 mg Al(OH)3
in 0.120 ml
+
65 mg Lysine
1.380 ml H2O
+
30 min Room T.
+
Centrifugation
8000rpm 10 min

Culot + dissolution in Citrate	Supernatant
below detection	**3.8**

Thus, Rotavirus may be liberated from the Rotavirus – $Al(OH)_3$ association and the liberated Rotavirus remains active.
This liberation can be done either by dissolving $Al(OH)_3$ (by HCl in the stomach, or by $Na_3Citrate$ in vitro) or by displacing Rotavirus by a basic amino acid (lysine).

5.7: Infectivity of the $Al(OH)_3$-Rotavirus association

A single dose of lyophilised Rotavirus was reconstituted with water and divided into two parts. The first part, considered as the reference, received an additional volume of water. The second part received 24mg of $Al(OH)_3$ suspended in 0.240 ml of water (Preclinical viral titrations).



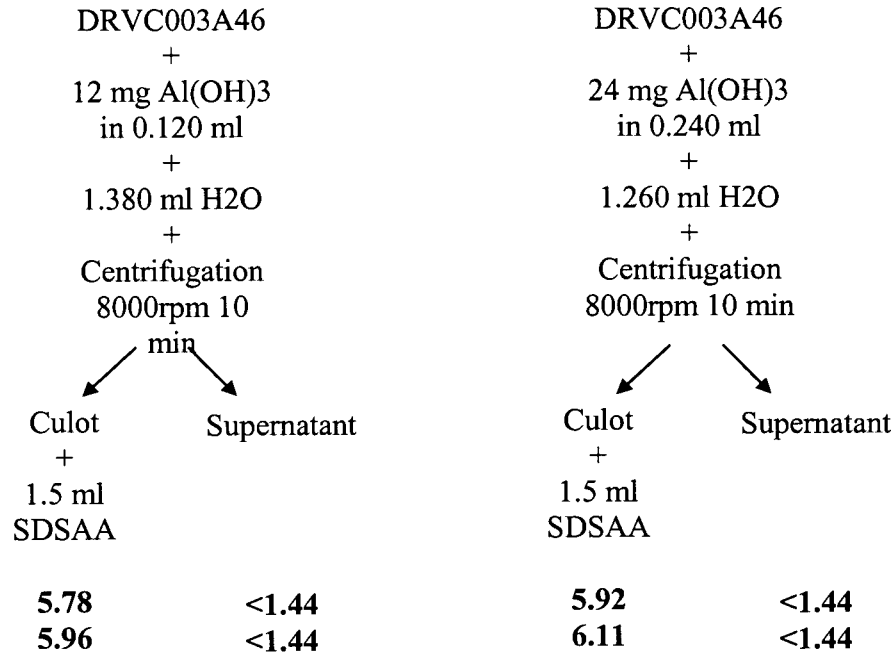
When $Al(OH)_3$ is present, Rotavirus is active and the viral titration value is higher compared to the reference sample.

This experiment was repeated without dividing the lyophilised dose, and by adding 12 mg $Al(OH)_3$ or 24 mg $Al(OH)_3$.

Here the reference sample was the one reconstituted with a Citrate-Bicarbonate buffer. Thus, the viral titer is again higher in the presence of $Al(OH)_3$.

DRVC003A46 + 1.5 ml WL buffer	DRVC003A46 + 12 mg Al(OH)3 in 0.120ml + 1.380ml H2O	DRVC003A46 + 24 mg Al(OH)3 in 0.240ml + 1.260ml H2O
5.34	**6.24**	**6.05**
5.32	**5.95**	**6.26**

As in the example above, Rotavirus associates with the $Al(OH)_3$ particles, since the virus can be discarded by centrifugation. DRVC003A46 is a lyophilised formulated Rotavirus (Sucrose:2%; Dextran: 4%,Sorbitol:3%; Amino-acids:2%).



SDSAA= Sucrose 2%, Dextran 4%, Sorbitol 3%, Amino-Acid 2%.

According to the viral titration carried out on the supernatant, the quantity of $Al(OH)_3$ needed to adsorb Rotavirus seems to be low (starting with one lyophilised dose 5.7 log) scaling Up viral titration):

Table 10

Al(OH)3	Adsorption time	Titer in supernatant
12 mg	1 hour RT	2.7
24 mg	1 hour RT	3.4
48 mg	1 hour RT	3.4
72 mg	1 hour RT	2.0
96 mg	1 hour RT	Below detection
12 mg	Overnight	2.7

24 mg	Overnight	Below detection
48 mg	Overnight	2.5
12 mg	Immediate	Below detection
24 mg	Immediate	2.0
48 mg	Immediate	Below detection

Time needed to adsorb Rotavirus on $Al(OH)_3$ seems to be short:

One dose of lyophilised Rotavirus was reconstituted in presence of 24 mg $Al(OH)_3$, and centrifuged after 0, 15, 60 min and 24 hours. The "culot" were resuspended in SDSAA before viral titration:

Table 11

Time	**Culot**	**Supernatant**
0 min	5.26	3.17
15 min	5.34	<1.44
60 min	5.96	<1.44
24 hours	6.13	<1.44

5.8: Using $CaCO_3$ as antacid

In order to avoid aluminium in the vaccine, the antacid $Al(OH)_3$ was replaced by another insoluble inorganic salt: $CaCO_3$ (calcium carbonate).

The phenomena observed with $CaCO_3$ are parallel to those described for $Al(OH)_3$:

- Association of Rotavirus with the inorganic salt;
- Maintainance of Rotavirus activity when associated with the inorganic salt;
- Possibility of liberation of Rotavirus from the association by dissolution of the inorganic base by an acid;
- Possibility of co-lyophilisation of the antacid and the Rotavirus.

$CaCO_3$ and Rotavirus association

In a first trial, lyophilised Rotavirus (viral titer 5.7) was reconstituted with a suspension of $CaCO_3$ in water (50mg in 1.5ml); and then centrifuged, and the viral titer of the supernatant compared to the pellet.

DRVC003A46 + 50mg CaCO3 in 1.5 ml H2O + Centrifugation 8000rpm 10 min

Culot + 1.5 ml SDSAA

5.83

Supernatant

4.46

DRVC003A46 + 50 mg CaCO3 in 1.5 ml H2O + Centrifugation 8000rpm 10 min

Culot + 1.5 ml Na Citrate

5.88

Supernatant

4.33

This indicates that more that 90% of the Rotavirus is associated with $CaCO_3$.

Also, when the virus was associated, it was possible to realise the titration and to recover the original viral quantities.

Also, viral titers are slightly higher that those obtained without $CaCO_3$.

Quantity of $CaCO_3$ and Rotavirus association

DRVC003A46 + 1.5 ml H2O + Centrifugation 8000rpm 10 min

"Culot"

4.99

Supernatant

5.03

DRVC003A46 + 1.5 ml W.L Buffer

5.35

Lyophilised Rotavirus was reconstituted with a $CaCO_3$ suspension in water (1.5ml):

10 mg

50 mg

100 mg

and then centrifuged, and the viral titer of the supernatant compared to the culot.

Table 12

CaCO3	Extempo + Centri.		1 Hour + Centri	
	Culots	Surpernatant	Culots	Surpernatant
100mg	4.57	3.01	4.79	3.09
50mg	4.17	4.15	4.22	3.86
10mg	3.17	4.77	3.87	4.87

Thus, clearly, more $CaCO_3$ and more virus is associated, and less is found in the supernatant.
However, the full dose is not completely recovered (expected a total of 5.3 at least or even 5.8 as obtained earlier – see above).

$CaCO_3$ Protection of Rotavirus during Baby Rossett-Rice antacid titration

Using 10 doses of lyophilised Rotavirus (DRVC003A46) and 50mg of $CaCO_3$, two types of baby Rossett-Rice titration were carried out:

In a classic Rossett-Rice titration, the antacid is mixed with Rotavirus and HCl is poured into this medium.

In the "inverse" baby Rossett-Rice, the situation is the reverse: antacid is dropped into the HCl pool (as it occurs in vivo).

Table 13

Classical baby Rossett-Rice titration			
Lyophi. Rota stored at:	Buffer	Theoretical Viral Titer	Measured Viral Titer
4°C	60 mg CaCO3	5.3	4.6
-80°C	60 mg CaCO3	5.3	4.6
4°C	24 mg Al(OH)3	5.4	<2.9

-80°C	24 mg Al(OH)3	5.4	<2.9

Inverse baby Rossett-Rice titration			
Lyophi. Rota stored at:	Buffer	Theoretical Viral Titer	Measured Viral Titer
4°C	60 mg CaCO3	5.3	4.6
-80°C	60 mg CaCO3	5.3	4.6
4°C	24 mg Al(OH)3	5.4	<2.9
-80°C	24 mg Al(OH)3	5.4	<2.9

Thus, in this *in vitro* experiment, calcium carbonate is able to protect about 20% of Rotavirus from the presence of HCl, while aluminium hydroxide is not able to.

5.9: Lyophilisation of Rotavirus in presence of $CaCO_3$ antacid:

Table 14

Batch n°	Composition	Viral titer at time = zero after lyophilisation	Viral titer after lyopjhilisation and 1 week at 37°C
99K08/01	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2% $CaCO_3$: 50 mg	$10^{5.28}$	$10^{5.10}$
99K08/02	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2% $CaCO_3$: 60 mg	$10^{5.16}$	$10^{5.15}$
00C24/01	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2% $CaCO_3$: 60 mg Xanthane 0.3%	$10^{5.07}$	$10^{4.69}$
00C24/03	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2% $CaCO_3$: 60 mg Xanthane 0.3%	$10^{5.07}$	$10^{4.85}$
00E09/25	Sucrose: 2% Dextran: 4%	$10^{5.03}$	$10^{4.91}$

	Sorbitol: 3% Am. Acids: 2% $CaCO_3$: 60 mg Xanthane 0.25%		
00E09/30	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2% $CaCO_3$: 60 mg Xanthane 0.30%	$10^{5.01}$	$10^{4.87}$
00F26/06	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2% $CaCO_3$: 60 mg Starch: 2%	$10^{4.50}$	$10^{4.70}$

This is the "all in one" - lyophilisation of Rotavirus and antacid (CaCO3) together in the same vial. To prevent sedimentation of $CaCO_3$ during the filling step, viscous agents are needed. Examples of such viscous agents include Xanthane gum and Starch. The Rotavirus activity is maintained even in the presence of Xanthane gum and Starch.

5.10 Lyophilised tablets for quick disintegration when placed in the mouth:

The following formulations demonstrate the "lyoc" concept. That is, quick dissolution of the lyophilised cake in the mouth.

Table 15

Batch n°	Formulation composition	Viral titer before lyophilisation	Viral titer after lyopjhilisation and 1 week at 37°
99B10/06	Sucrose 4% Sodium glutamate 3.7% Al(OH)3 48mg	$10^{5.11}$	$10^{4.53}$
99C11/12	Maltitol 3% Al(OH) 48mg Hydroxypropylmethyl-cellulose: 1%	$10^{4.16}$	$10^{3.79}$

Batch n°	Fomulation composition	Viral titer at time = zero after lyophilisation	Viral titer after lyopjhilisation and 1 week at 37°

00C24/05	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2% $CaCO_3$: 60 mg Xanthane 0.3%	$10^{5.02}$	$10^{4.54}$
00C24/06	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2% $CaCO_3$: 60 mg Xanthane 0.3%	$10^{4.86}$	$10^{4.56}$
00F26/11	Sucrose: 1% Dextran: 2% Sorbitol: 1.5% Am. Acids: 1% $CaCO_3$: 60 mg Starch: 2%	$10^{4.70}$	$10^{4.40}$

In the "lyoc concept" both Xanthane and Starch can be used (maintaining the quick dissolution properties of the lyophilised cake).

EXAMPLE 6: Use of Calcium Carbonate as the antacid for the Rotavirus vaccine composition

When a suspension of $CaCO_3$ in water is used as the antacid for Rotavirus there is a problem that the calcium carbonate particles sediment rapidly when placed in water since the powder density value approaches 2.6 and the average particle size is 30µm. This sedimentation can be slowed by:

1 increasing the density of the surrounding medium
2 increasing the viscosity of the surrounding medium
3 reducing the particles size
4 keeping particles away from each other

6.1: Increasing density of the surrounding medium:

When the $CaCO_3$-Water suspension (when placed in the syringe) is placed on the lyophilised cake (containing sucrose 2%, dextran 4%; sorbitol 3%; amino-acids 2%) the

density of the surrounding medium is increased, but the speed of $CaCO_3$ sedimentation is not very much different from the $CaCO_3$-Water suspension.

6.2 Increasing the viscosity of the surrounding medium:

Pseudoplastic excipents

A pseudoplastic solution is defined as a solution having higher viscosity on standing compared to its viscosity under agitation.

Usual excipients of this type are:

natural polymers for example:

- arabic gum
- adragante gum
- agar-agar
- alginates
- pectines

semi-synthetic polymers for example:

- carboxymethylcellulose (Tyloses C®)
- methylcellulose (Methocels A®, Viscotrans MC®, Tylose MH® and MB®)
- hydroxypropylcellulose (Klucels®)
- hydroxypropylmethylcellulose (Methocels E® and K®, Viscontrans MPHC®)

In general those pseudoplastic excipients are used together with thixotropic agents.

Pseudoplastic excipients with low flowing capacity

Those polymers, at a sufficient concentration, give rise to a structural fluid arrangement resulting in a high viscosity solution having low flowing capacity on standing. A certain quantity of energy needs to be given to the system to allow flowing and transfer. External energies (agitation) are needed to destroy temporarily the structural fluid arrangement in order to obtain a fluid solution.

Examples of such polymers are Carbopols® and Xanthane gum.

Thixotropic excipents

With these excipents, on standing, a gel structure is obtained; while under agitation a fluid solution is obtained.

Examples of thixotropic excipients are: Veegum ®(Magnesium-aluminium silicate) and Avicel RC® (about 89% microcrystalline cellulose and 11% Caboxymethylcellulose Na).

6.3 Reducing the particles size

A reduction in the $CaCO_3$ particle size resulted in a decrease in the antacid capacity of the compound.

6.4 Keeping particles away from each other

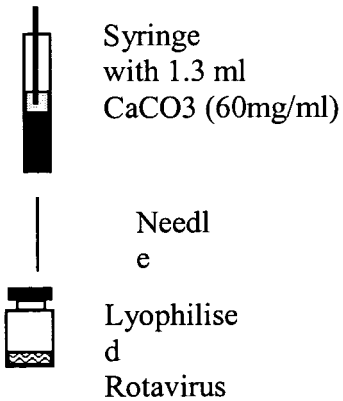
This is the case in Veegum® and Avicel® for which insoluble particles smaller (about 1 µm) than the $CaCO_3$ particles, are placed between $CaCO_3$ particles in order to prevent aggregation.

EXAMPLE 7: Product design

The following schemes demonstrate examples of possible product designs.

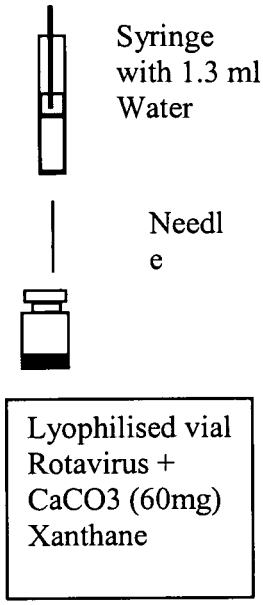
7. 1 $CaCO_3$ in the syringe

Having already clinical batches of Rotavirus in lyophilised vials, the antacid can be placed in the reconstituent liquid contained in the syringe.



In this product presentation, sedimentation of $CaCO_3$ must be under control not only during the filling steps, but also during the complete shelf-live of the product (at least 2 years).

7. 2 $CaCO_3$ in the lyophilised vial



7.3. Lyophilisation in a blister

In this case Rotavirus, $CaCO_3$ and Xanthane gum are lyophilised together directly in the blister.



EXAMPLE 8: Lyophilisation of different strain of Rotavirus

Table 16

Batch n°	Rotavirus strain	Fomulation composition	Viral titer at t = zero after lyophilisation	Viral titer after lyopjhilisation and 1 week at 37°
00F26/01	G1 SB purif n°61	Sucrose: 2% Dextran: 4%	$10^{4.6}$	$10^{4.7}$

	PRO/0232	Sorbitol: 3% Am. Acids: 2%		
00F26/02	G2 (DS-1)	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2%	$10^{4.4}$	$10^{4.4}$
00F26/03	G3(P)	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2%	$10^{4.6}$	$10^{4.5}$
00F26/04	G4 (VA-70)	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2%	$10^{4.8}$	$10^{4.8}$
00F26/05	G9 (W161)	Sucrose: 2% Dextran: 4% Sorbitol: 3% Am. Acids: 2%	$10^{4.6}$	$10^{4.5}$

The strains DS-1, P and VA70 are described as Human rotavirus reference strains for serotype G2, G3 and G4 respectively at page 1361 of "Fields" Raven press 1990, second edition.

In this experiment different Rotavirus strains have been lyophilised.
For all, both the viral titer have been maintained during lyophilisation and accelarated stability (one week at 37°C) has been shown.

EXAMPLE 9: Phase I safety study in adults of one oral administration of the Rotavirus vaccine.

A Phase I study was carried out to assess the safety and reactogenicity of a single oral dose of $10^{6.0}$ ffu of the P43 vaccine in healthy adults aged 18 to 45 years.
The clinical trial was double blind and randomized. It was placebo-controlled and self-contained. The study was performed in one single centre in Belgium.

9.1. Study Population

A total of 33 subjects, 11 in the placebo group and 22 in the vaccine group, were enrolled and all completed the study. All volunteers were Caucasians. Their mean age at the time of vaccination was 35.3 years, with a range of 18 to 44 years. The trial began in January and ran for just over one month.

9.2. Material

Vaccine
Clinical lots of P43 vaccine were produced, purified, formulated and lyophilized according to Good Manufacturing Practices. The lots were released by Quality Control and Quality Assurance. Each vial of vaccine contained the following components:

Active ingredient:

P43 strain	Min. $10^{5.8}$ ffu

Excipients, stabilizers:

Sucrose	9 mg
Dextran	18 mg
Sorbitol	13.5 mg
Amino acids	9 mg

Placebo
Vials of placebo were prepared and released. Each vial of placebo contained the following components:

Excipients, stabilizers:

Sucrose	9 mg
Dextran	18 mg
Sorbitol	13.5 mg
Amino acids	9 mg

Diluent
Water for injection was used as diluent to reconstitute vaccine and placebo.

9.3. Administration

Approximately 10 to 15 minutes before administration of the vaccine or the placebo, subjects of both groups were given 10 ml of Mylanta® orally. Mylanta® is a registered antacid. The antacid increases the pH of the stomach and prevents inactivation of the rotavirus during its passage through the stomach.
To prepare the vaccine, two vials of lyophilized P43 containing $10^{5.8}$ ffu per vial were reconstituted with 1.5 ml of diluent water for injection. This achieved a calculated viral

titer of $10^{6.1}$ ffu per dose. The reconstituted vaccine was administered promptly as a single oral dose.

To prepare the placebo, two vials of lyophilized placebo were reconstituted with 1.5 ml water for injection and administered orally as a single dose.

9.4. Safety and Reactogenicity

The following criteria of safety and reactogenicity applied:

Solicited general symptoms were fever, diarrhea, vomiting, nausea, abdominal pain and loss of appetite. They were recorded during eight days post administration.

Unsolicited symptoms were recorded during 30 days post administration.

Serious adverse events were recorded during the entire study period.

Diarrhea samples were to be collected during eight days post administration.

The results were:

No solicited symptoms, no unsolicited and no serious adverse events were reported during the respective observation periods.

No cases of diarrhea were reported.

9.5. Conclusions

SB Biologicals P43 vaccine was safe relative to the placebo when administered orally in a double-blind fashion as a single dose at the dose of $10^{6.1}$ ffu to healthy adult volunteers aged 18 to 44.

EXAMPLE 10 - Efficacy of two doses of a human monovalent Rotavirus vaccine, containing RIX 4414 in preventing Gastro-enteritis due to G1 and non-G1 (G9) Rotavirus

10.1. Methods

A randomised, double-blind, placebo-controlled phase II trial was conducted in Latin America to evaluate the protective efficacy of a vaccine (RIX4414 human rotavirus strain) derived from the G1P[8] human strain 89-12 for infant immunisation. RIX4414 vaccine comprises as rotavirus component the attenuated G1P[8] human strain deposited as ECACC deposit 99081301 (WO 01/12797).

Vaccine composition (Table 17)

The HRV vaccine or placebo was prepared for administration by injecting the entire content of one pre-filled syringe containing the calcium carbonate buffer into the vial of the lyophilized product (vaccine or placebo). The vial was shaken to resuspend the vaccine/placebo. The entire volume of the resuspended product was withdrawn into the same syringe, the needle discarded and the resuspended product administered promptly as a single oral dose (approximately 1.0 ml).

Table 17 – RIX4414 rotavirus vaccine composition

Ingredients		**Quantity (per nominal dose: 1 ml)**
Active ingredient		
RIX4414		$10^{5.8}$ ffu/dose
Excipients		
Lyophilized vaccine in glass vial	Sucrose	9 mg
	Dextran	18 mg
	Sorbitol	13.5 mg
	Amino acids	9 mg
	Dulbecco's Modified Eagle Medium (DMEM)	2.25 mg
Liquid diluent ($CaCO_3$-based) in pre-filled syringe		
	Calcium carbonate	60 mg
	Xanthan	2.5 mg
	Water for Injections	q. s. ad 1 ml

Vaccine administration

Healthy infants (493) received two doses of the RIX4414-rotavirus vaccine at a viral concentration of $10^{5.8}$ ffu per dose, or placebo (504) at age 2 and 4 months, concomitantly with DTPw-HBV and Hib vaccines. Three doses of OPV (oral polio virus vaccine) were given 2 weeks apart from study vaccine, i.e. were not to be administered during the period starting 2 weeks before each dose of study vaccine and ending 2 weeks after. Two other groups received 2 doses of the RIX4414-rotavirus vaccine at different viral concentrations: $10^{4.7}$ ffu and $10^{5.2}$ ffu. Diarrhoeal samples were tested for the presence of rotavirus (ELISA) and the serotypes determined in positive samples (RT-PCR). Diarrhoeal episodes reported from two weeks after the second dose were considered for the efficacy analysis. Severity was determined using a 20-point scale

(Ruuska and Vesikari, 1990). The 20-point scoring system used to assess the severity of each diarrhoea episode in this study is shown below in Table 18. A score ≥ 11 defined severe disease.

Table 18

Adverse Experience	Points
Duration of looser than normal stools (days)	
1–4	1
5	2
≥ 6	3
Maximum number of looser than normal stools/24 hours	
1–3	1
4–5	2
≥ 6	3
Duration of vomiting (days)	
1	1
2	2
≥ 3	3
Maximum number of episodes of vomiting/24 hours	
1	1
2–4	2
≥ 5	3
Fever (measured rectally/axillary)*	
37.1–38.4°C/36.6-37.9°C	1
38.5–38.9°C/38.0-38.4°C	2
≥ 39°C/≥ 38.5°C	3
Treatment	
Rehydration	1
Hospitalization	2
Dehydration	
1–5 %	2
≥ 6%	3

* The highest temperature recorded during the episode was scored.

10.2. Results

An interim analysis of efficacy was performed on the above mentioned group and the isolated serotypes were mainly G1 and G9, almost evenly distributed. The overall attack rate in the placebo group varied from 4.8% for G1 to 3.6% for G9 during the 6 months observation period. Two doses of RIX4414 rotavirus vaccine at $10^{5.8}$ ffu protected against all types of diarrhoea caused by G1 with 83% efficacy [95% CI: 50.4-95.7] and 92.1 % efficacy [95% CI: 47.6-99.8] against severe gastro-enteritis. If the diarrhoea was

caused by G9, the protection against all types of diarrhoea was 60.2 % [95% CI: 0.2-86.0] and 80.8% [95% CI: 33.0-96.4] against severe gastro-enteritis. For each of these efficacy endpoints (any and severe for G1 and G9), there was a statistically significant decrease in diarrhoea episodes in the HRV group as compared to the placebo group ($p < 0.05$, two-sided Fisher's exact test).

The results obtained in the other 2 vaccine groups (different rotavirus concentration) are consistent with those reported in the Example, and are presented in the final analysis (Example 11). Efficacy data for G2, G3 and G4 were also analysed. No conclusion from this study was drawn about G2, G3 and G4 cross-protection as too few cases were reported. However data of efficacy against G2, G3 and G4 are presented in the final analysis on a more important sample size (Example 11).

10.3. Conclusion.

These results are highly supportive of the efficacy of 2 doses of a monovalent HRV vaccine, RIX4414 rotavirus vaccine in protecting young infants against G1 strain and cross-protect against the G9 strain.

EXAMPLE 11 - Efficacy of two doses of a human monovalent Rotavirus vaccine, containing RIX4414 strain, administered at three different virus concentrations in preventing Gastro-enteritis due to G1 and non-G1 (G2, G3, G4, G9) Rotavirus

11.1. Methods

A randomised, double-blind, placebo-controlled phase II trial was conducted in Latin America to evaluate the protective efficacy and efficacy against hospitalization of a vaccine derived from the G1P[8] human strain 89-12 for infant immunisation. Specifically the vaccine used was named RIX4414 rotavirus vaccine, and comprises as the rotavirus component the attenuated G1 human strain deposited as ECACC deposit 99081301.

Healthy infants received two doses of RIX4414 rotavirus vaccine at three different virus concentrations. The cohort for efficacy analysis consisted of 1846 subjects (468 subjects in the $10^{4.7}$ ffu HRV vaccine group, 460 subjects in the $10^{5.2}$ ffu HRV vaccine group, 464 subjects in the $10^{5.8}$ ffu HRV vaccine group and 454 subjects in the placebo group at age 2 and 4 months, concomitantly with DTPw-HBV and Hib vaccines. Three doses of OPV were given 2 weeks apart from study vaccine, i.e. were not to be administered during the

period starting 2 weeks before each dose of study vaccine and ending 2 weeks after. Diarrhoeal samples were tested for the presence of rotavirus (ELISA) and the serotypes determined in positive samples (RT-PCR). Diarrhoeal episodes reported from two weeks after the second dose until subjects were one year of age were considered for the efficacy analysis. Severity was determined using a 20-point scale (Ruuska and Vesikari, 1990). A score ≥ 11 defined severe disease (see Example 10 for the description of the 20-point scoring system).

11.2. Results

Results which are the final analysis of the data mentioned in Example 10 are illustrated in the tables below. Infants in the vaccine groups had significantly fewer rotavirus gastroenteritis episodes than children in the placebo group ($p < 0.001$, two-sided Fisher's exact test) (Table 19). Depending on the dosage, protective efficacy against severe rotavirus gastroenteritis reached 85.6% (95%CI: 63.0%-95.6%), and 70% (95%CI, 45.7%-84.4%) against any rotavirus gastroenteritis (Table 20). For each of these efficacy endpoints, there was a statistically significant decrease in diarrhoea episodes in the HRV group as compared to the placebo group ($p < 0.001$, two-sided Fisher's exact test). Multiple rotavirus serotypes (G1, G2, G3, G4 and G9) were identified from gastroenteritis stools (ELISA and RT-PCR) allowing to also calculate vaccine efficacy against non-G1 serotypes. As can be seen from Table 21 in particular, for non-G1 serotypes (G2, G3, G4 and G9), and depending on the dosage, efficacy against severe rotavirus gastroenteritis reached 82.7% (95%CI: 40.3%-96.8%), providing proof of concept that the monovalent G1-based G1P1A P[8] human rotavirus vaccine elicits cross-protection against heterotypic (i.e. non-G1 and non-P[8]) strains.

Table 19: Features of rotavirus gastro-enteritis episodes reported during the study

		RIX4414 $10^{4.7}$ ffu	RIX4414 $10^{5.2}$ ffu	RIX4414 $10^{5.8}$ ffu	Placebo
Any rotavirus gastroenteritis		21	22	15	51
no. of episodes (percent) with specific feature among all rotavirus gastroenteritis episodes reported					
Severity scores	<7	4 (19.0)	8 (36.4)	2 (13.3)	5 (9.8)
	7-10	5 (23.8)	4 (18.2)	8 (53.3)	12 (23.5)
	≥ 11	12 (57.1)	10 (45.5)	5 (33.3)	34 (66.7)

Identified rotavirus serotypes					
	wild G1	12 (57.1)	6 (27.3)	7 (46.7)	30 (58.8)
	G2	0	0	1 (6.7)	3 (5.9)
	G3	1 (4.8)	0	0	2 (3.9)
	G4	0	0	1 (6.7)	0
	G9	8 (38.1)	14 (63.6)	7 (46.7)	15 (29.4)
	Canine	0	0	0	1(2.0)
	Unknown	0	2 (9.1)	0	0

Table 20: Protective efficacy of two doses of RIX4414 human rotavirus vaccine against rotavirus gastroenteritis

		Any rotavirus gastroenteritis		Severe rotavirus gastroenteritis		Hospitalization for rotavirus gastroenteritis	
	N	**n (%)**	**Efficacy (95% CI)**	**n (%)**	**Efficacy (95% CI)**	**n (%)**	**Efficacy (95% CI)**
Pooled vaccine groups	1392	58 (4.2)*	61.4 (42.3;4.1)	27 (1.9)*	74.1 (55.8;85.0)	9 (0.6) *	79.0 (48.0;92.0)
RIX4414 $10^{5.8}$ ffu	464	15 (3.2)*	70.0 (45.7;84.4)	5 (1.1)*	85.6 (63.0;95.6)	3 (0.6)‡	79.0 (24.9;96.1)
RIX4414 $10^{5.2}$ ffu	460	22 (4.8)*	55.7 (25.3;74.5)	10 (2.2)*	71.0 (39.9;87.2)	1 (0.2)*	93.0 (53.7;99.8)
RIX4414 $10^{4.7}$ ffu	468	21 (4.5)*	58.4 (29.4;76.3)	12 (2.6)*	65.8 (32.2;83.9)	5 (1.1)†	65.4 (-1.8;90.2)
Placebo	454	49 (10.8)	-	34 (7.5)	-	14 (3.1)	-

*p<0.001 for each comparison between the vaccine and placebo groups by two-sided Fisher's exact test (significant level of α=0.05)

†p=0.037 for the comparison between the vaccine and placebo groups by two-sided Fisher's exact test (significant level of α=0.05)

‡p=0.007 for the comparison between the vaccine and placebo groups by two-sided Fisher's exact test (significant level of α=0.05)

N = number of subjects

n/% = number/percentage of subjects reporting at least one specified rotavirus gastroenteritis episode

Exact 95% confidence intervals are shown

Table 21: Protective efficacy of two doses of RIX4414 human rotavirus vaccine against serotype specific severe rotavirus gastroenteritis

		Severe rotavirus gastroenteritis		
	N	n(%)	Efficacy (95% CI)	p-value*
G1 wild type rotavirus				
Pooled vaccine groups	1392	13 (0.9)	73.5 (41.2;88.3)	<0.001
RIX4414 $10^{5.8}$ ffu	464	2 (0.4)	87.8 (48.0;98.6)	<0.001
RIX4414 $10^{5.2}$ ffu	460	4 (0.9)	75.3 (23.5;94.0)	0.006
RIX4414 $10^{4.7}$ ffu	468	7 (1.5)	57.6 (-9.0;85.2)	0.057
Placebo	454	16 (3.5)	-	-
Non-G1 rotavirus (mainly G9 with G2, G3 and G4 types)				
Pooled vaccine groups	1392	14 (1.0)	73.1 (42.1;87.7)	<0.001
RIX4414 $10^{5.8}$ ffu	464	3 (0.6)	82.7 (40.3;96.8)	0.001
RIX4414 $10^{5.2}$ ffu	460	6 (1.3)	65.2 (7.4;88.8)	0.020
RIX4414 $10^{4.7}$ ffu	468	5 (1.1)	71.5 (19.4;91.8)	0.009
Placebo	454	17 (3.7)	-	-

*Two-sided Fisher's exact test (significant level of α=0.05) used for each comparison between the vaccine and placebo groups.
N = number of subjects
n/% = number/percentage of subjects reporting at least one specified severe rotavirus gastroenteritis episode
Exact 95% confidence intervals are shown

11.3. Conclusion

These results are highly supportive of the efficacy of 2 doses of a monovalent HRV vaccine containing RIX4414, in protecting young infants against any and severe rotavirus gastroenteritis caused by the G1 strain and broad cross-protection against other RV G types, namely G2, G3, G4 and G9.

EXAMPLE 12 – Two doses of the Human Attenuated Rotavirus vaccine RIX4414 vaccine show heterotypic protection in Latin America and Europe

The Efficacy of a 2 dose oral, live attenuated G1P[8] human rotavirus (RV) vaccine containing RIX 4414 strain was analysed in a Phase II/III clinical trials in Finnish and

Latin American infants. RIX 4414 rotavirus vaccine comprises as the rotavirus component the attenuated G1 human strain deposited as ECACC deposit 99081301.

12.1. Methods

Part of the results of Example 12 is already presented in Examples 10 and 11. Data were pooled from Phase II studies, one in Finland and one in Latin America (Brazil, Mexico and Venezuela) (Examples 10 and 11) and from one Phase III study in 11 Latin American countries (Example 13) using the same methodology and efficacy criteria. In total, 20081 healthy infants (cohort for efficacy) vaccinated with 2 doses of RIX 4414 vaccine or placebo at 2 and 4 months of age were followed until one year of age for severe gastroenteritis (GE) with a score on the Vesikari (Ruuska T et al. Scand. J. Infect. Dis. 1990, 22, 259-267) severity scale ≥ 11. GE samples were tested for rotavirus (by ELISA) and typed by RT-PCR.

A meta analysis was conducted on the three mentioned studies. Pooled efficacy for severe RV GE (defined as Vesikari severity score ≥ 11) was calculated from 2 weeks post-dose 2 to 1 year of age (adjustment for study effect using the Mantel-Haenszel approximation).

12.2. Results

In the cohort for efficacy 5 severe rotavirus GE episodes of G2P[4] type with a Vesikari score ≥ 11 were detected in the vaccine group and 13 episodes in the placebo group. Vaccine efficacy against the G2P[4] type was 67.2% (95% CI: 14.8; 87.1), which shows that in addition to protecting against homotypic strains (G1P[8], G3P[8] and G4P[8]), RIX4414 vaccine protects against severe rotavirus GE caused by the heterotypic non-P[8] non-G1 G2P[4] strain.

Type-specific efficacy across the different studies is given below (Table 22).

Table 22

Strain	Number of severe RV cases		% Vaccine Efficacy* (VE) (95% CI)
	N Vaccinees (N = 10646)	N Placebo (N = 9435)	

G1	17	52	83.7 (70.0; 91.2)
G2P[4]	5	13	67.2 (14.8; 87.1)
G3	2	8	82.7 (5.9; 96.8)
G9	15	34	79.5 (59.2; 89.7)

*VE adjusted for study effect using Mantel-Haenszel approximation

Only 3 G4 cases occurred in all 3 trials, 1 in vaccine and 2 in placebo recipients.

12.3. Conclusion

This analysis shows that, in addition to giving a high level of protection against homologous G1 rotavirus strains (which have two outer capsid proteins (VP4 and VP7) and one inner capsid protein (VP6) antigenically similar to the vaccine), RIX 4414 vaccine is also highly protective against other strains which have either a different G type (eg G3, G9), a different P type (eg P[4]), or both different G type and P type, as illustrated by the efficacy against G2P[4].

EXAMPLE 13 – Meta analysis showing that two doses of the Human Attenuated Rotavirus vaccine RIX 4414 show heterotypic protection

As more data became available from Singapore and from a European study (Example 15), an additional meta analysis was carried out to include these studies in addition to studies mentioned in Example 12.

13.1. Methods

Three phase II (Finland and Latin America and Singapore) and two phase III studies (Latin America and Europe) were included in the meta-analysis. Two oral doses were administered according to 0,1 to 2 month schedule to healthy infants who were 6-14 weeks of age at Dose 1. In all studies, severe RVGE was defined as a score ≥11 on the 20-point Vesikari scale. Diarrhoeal samples were analyzed for the presence of RV by ELISA and typed by RT-PCR based method. Efficacy against any RV GE was evaluated in the three phase II studies and the phase III Europe study only as in the phase III Latin America study, only severe RV GE were recorded.

VE and its 95%CI was estimated as 1–rate of RVGE relative to placebo using exact Poisson rate ratio stratified by study (Proc StatXact4 for SAS Users, 1999, cytel software corporation, exact Confidence Interval for common relative risk, p298)

13.2. Results

In a total of 8221 infants vaccinated with two doses of RIX4414 or placebo, 4 episodes of any G2P[4] RVGE were detected in the RIX4414 (N=5783) and 9 episodes in the placebo (N=2438) group, indicating a VE of 81.0% (95% CI:31.6;95.8) against RVGE of any severity due to G2P[4] strain.

In a total of 26088 healthy infants vaccinated with two doses of RIX4414 or placebo, 6 episodes of severe RVGE due to G2P[4] type were detected in the RIX4414 (N=14792) and 15 episodes in the placebo (N=11296) group, indicating a VE of 71.4% (95% CI:20.1;91.1) against severe RVGE due to G2P[4] strain. Results are reported in Table 23.

Table 23 - Number of subjects reporting any or severe RV GE episodes caused by G2P[4] RV type and percentage of vaccine efficacy during the first efficacy period – (meta analysis), cohort for efficacy

Table 23

Groups		**Any RV GE* (Latin America excluded)**				**Severe RV GE (score ≥11 on Vesikari scale) (5 studies)**				
	N	n	%	Vaccine efficacy		N	n	%	Vaccine efficacy	
RV Strain				% VE	95% CI				% VE	95% CI
G2P[4]										
HRV vaccine	5783	4	0.07	**81.0**	31.6-95.8	14792	6	0.04	**71.4**	20.1-91.1
Placebo	2438	9	0.37			11296	15	0.13		

N = number of subjects included in each group;
n/% = number/percentage of subjects reporting at least one specified RV G2P[4] GE episode in each group;
% VE = observed vaccine efficacy,
95%CI = 95%Confidence Intervals
* Two out of 13 G2 were not P typed

13.3. Conclusion:

This meta analysis on vaccine efficacy against G2P[4] RV type shows a vaccine efficacy of 81.0% (95% CI: 31.6%; 95.8%) against any RV GE due to G2P[4] type and a vaccine efficacy of 71.4% (95% CI: 20.1%; 91.1%) against severe RV GE due to G2P[4] type.

EXAMPLE 14 – Efficacy of Human Attenuated Rotavirus vaccine Rotarix™ in a multi-country phase III trial

14.1. Methods

20169 healthy infants from 11 Latin American countries were to receive two oral doses of HRV vaccine (10159) or placebo (10010) at approximately 2 and 4 months of age. Stool specimens were tested for rotavirus (RV) by ELISA and typed by RT-PCR using suitable primers and type-specific probes. The clinical case definition for capture of severe gastroenteritis episode was an episode of diarrhea (passage of three or more looser than normal or watery stools within 24 hours) with or without vomiting that required overnight hospitalization and/or rehydration therapy equivalent to WHO plan B (oral rehydration therapy) or WHO plan C (intravenous rehydration therapy) in a medical facility such as hospital, clinic or supervised rural health care center (http://www.who.int/child-adolescent-health/New_Publications/CHILDHEALTH/textrev4.htm). Disease severity was graded using the 20-point Vesikari scale; severe RVGE was defined as a score ≥11. Vesikari's score was modified: Since the dehydration was not recorded in the eCRF, the following rule was applied: a subject that had a severe GE episode was considered as being dehydrated between 1 to 5% if this subject received oral re-hydration. A subject was considered as being dehydrated ≥ 6 % if the subject was hospitalized and/or received intravenous (IV) re-hydration.

14.2. Vaccine efficacy

Vaccine efficacy against severe rotavirus gastroenteritis (Table 24)

The cohort for efficacy consisted of 9009 subjects vaccinated with HRV vaccine and 8858 subjects receiving a placebo recipient. There were 12 children with severe rotavirus gastroenteritis according to the clinical definition in the vaccine and 77 in the placebo group (2.0 *vs.* 13.3 children with ≥1 episode per 1,000 child-years, respectively; $p<0.001$, two-sided Fisher's exact test), resulting in a vaccine efficacy of 84.7% against

severe rotavirus gastroenteritis from 15 days post-dose 2 until one year of age (shown in Table 24). Similar results were obtained with the total vaccinated cohort (vaccine efficacy of 81.1%; 95 % C.I. 68.5-89.3; p< 0.001, two-sided Fisher's exact test) from dose 1 until one year of age. Hospitalization for at least one night was required in 9 children in the vaccine and 59 in the placebo group (1.5 *vs.* 10.2 hospitalizations per 1,000 child-years, respectively), for a vaccine efficacy against hospitalization for severe rotavirus gastroenteritis of 85 % (p<0.001, two-sided Fisher's exact test) (Table 24).

Table 24 - Vaccine efficacy against rotavirus severe gastroenteritis, specific rotavirus G types severe gastroenteritis and all-cause severe gastroenteritis, during the period from two weeks after dose 2 until one year of age

	Vaccine group (N = 9,009)		Placebo group (N = 8,858)		RR	Vaccine efficacy (95% CI) and p_values
	n	1,000 infants-year ratio	n	1,000 infants-year ratio		
Severe rotavirus gastroenteritis according to the clinical case definition*						
Rotavirus gastroenteritis						
Severe	12	2.0	77	13.3	0.153	84.7 (71.7; 92.4) <0.001
Hospitalization	9	1.5	59	10.2	0.150	85.0 (69.6; 93.5) <0.001
All-cause gastroenteritis						
Severe	183	30.9	300	51.7	0.600	40.0 (27.7; 50.4) <0.001
Hospitalization	145	24.5	246	42.4	0.580	42.0 (28.6; 53.1) <0.001
Type specific gastroenteritis						
G1P[8]#	3[a]	0.5	36[b]	6.2	0.082	91.8 (74.1; 98.4) <0.001
G3P[8], G4P[8], G9P[8]	4[c]	0.66	31[d]	5.3	0.126	87.3 (64.1;96.7) <0.001
G2P[4]	6	1.0	10[e]	1.7	0.590	41.0 (-79.2; 82.4) 0.328
Severe rotavirus gastroenteritis with a score ≥ 11 on the Vesikari scale						

	Vaccine group (N = 9,009)		Placebo group (N = 8,858)		RR	Vaccine efficacy (95% CI) and p_values
	n	1,000 infants-year ratio	n	1,000 infants-year ratio		
Type specific gastroenteritis						
G1P[8][#]	3	0.5	32	5.5	0.092	90.8 (70.5;98.2) <0.001
G3P[8], G4P[8], G9P[8]	4	0.66	30	5.2	0.130	86.9 (62.8;96.6) <0.001
G2P[4]	5	0.8	9	1.5	0.546	45.4 (-81.5;85.6) 0.298

Legend to Table 24:

Participants with episodes with more than one isolated G type were counted in each of the detected rotavirus type category.
n = number of infants reporting at least one specified episode
RR = Relative Risk = ratio of the incidence rate of subjects reporting at least one episode in the vaccine group over the incidence rate of subjects reporting at least one episode in the placebo group.
CI = confidence interval
The 1000-infant year ratio is the number of infants presenting with >= 1 specified episode per infant-year
*Case definition according to the study protocol: an episode of diarrhea (passage of three or more looser than normal or watery stools within a day) with or without vomiting that required overnight hospitalization and/or rehydration therapy equivalent to WHO plan B (oral rehydration therapy) or WHO plan C (intravenous rehydration therapy) in a medical facility such as hospital, clinic or supervised rural health care center.
[#] All G1 types isolated were wild-type rotavirus; G1P[8] and G9P[8] were isolated from one infant
[a] G1P[8] type alone was isolated from 2 infants; G1P[8] and G9P[8] were isolated from one infant
[b] ; G1P[8] type alone was isolated from 34 infants; G1P[8] and G9P[8] were isolated from one infant; G1, G2, G9 types were isolated from one infant
[c] G3P[8] type alone was isolated from one infant, G4P[8] type alone from 1 infant; and G9P[8] alone from one infant; G1P[8] and G9P[8] were isolated from one infant
[d] G3P[8] type alone was isolated from 8 infants, G4P[8] type alone from 2 infants; and G9P[8] alone from 19 infants; G1P[8] and G9P[8] were isolated from 1 infant; and G1P[8] and G2P[4 and G9P[8] from 1 infant
[e] G2P[4] alone was isolated from 9 infants and G1P[8], G2P[4] and G9P[8] were isolated from 1 infant

p_values =two-sided Fisher's exact test (significant level of α=0.05)

Vaccine efficacy according to Vesikari score

Eleven of 12 children with severe rotavirus episodes in the vaccine group and 71 of 77 in the placebo group had Vesikari score ≥11, resulting in a vaccine efficacy of 84.7%t (P<0.001, two-sided Fisher's exact test). For increasing disease severity with scores between 11 and 20, vaccine efficacy was increasingly higher, reaching 100% against more severe rotavirus gastroenteritis. A total of 16 severe rotavirus gastroenteritis episodes with Vesikari score ≥11 were reported from dose 1 until dose 2, six in the vaccine group and 10 in the placebo group.

Vaccine efficacy according to Vesikari score per rotavirus type

Type specific vaccine efficacy against wild-type strains is shown in Table 24. Vaccine efficacy against severe rotavirus episodes with Vesikari score ≥11 caused by G1P[8] type strains, homologous to the vaccine strain, was 91.8% (P<0.001, two-sided Fisher's exact test). Vaccine efficacy against strains sharing the P[8] antigen (G3P[8], G4P[8] and G9P[8]) was 86.9% (P<0.001, two-sided Fisher's exact test). G2P[4] rotavirus type, which is not sharing either the G nor the P antigen with the vaccine strain was detected in five episodes in the vaccine and nine in the placebo group, resulting in an efficacy of 45 percent (P=0.298, two-sided Fisher's exact test). Because of the small number of G2 episodes observed in this study, a meta-analysis of 5 studies (Example 13) was performed and the trend observed in this study has become a significant value when the results of the 5 studies were pooled. (Example 13)

Vaccine efficacy on the burden of diarrhea illness

Children with gastroenteritis of any cause requiring hospitalization and/or rehydration according to WHO plan B/C had an incidence rate of 30.9/1,000 child-years in the vaccine compared to 51.7 in the placebo group, for an overall 40 % (P<0.001, two-sided Fisher's exact test) reduction in severe diarrhea episodes of all cause among vaccine recipients. Likewise, hospitalization for diarrhea of any etiology was significantly reduced by 42 % (P<0.001, two-sided Fisher's exact test) (Table 24, all causes GE)).

14.3. Summary of results

Vaccine efficacy against severe rotavirus gastroenteritis (RV GE) and against rotavirus associated-hospitalization was 85% (P<0.001, two-sided Fisher's exact test), reaching 100% in a population having RV GE with a Vesikari's score >=19. Efficacy against G1P[8] and strains sharing only the P[8] epitope with HRV was 92% (95%C.I. 74,98) and

87% (95%C.I. 64,97) respectively (P<0.001, two-sided Fisher's exact test). Hospitalization for diarrhea of all cause was reduced by 42% (95%C.I. 29,53; P<0.001, two-sided Fisher's exact test).

Example 15 – Efficacy of Human Attenuated Rotavirus vaccine Rotarix™ in six European countries

15.1. Methods

3,994 children in six European countries were randomized to receive $10^{6.5}$ $CCID_{50}$ HRV (human rotavirus) vaccine Rotarix™ (see composition) or placebo when co-administered with routine childhood vaccinations. The first efficacy follow-up period started from two weeks after Dose 2 and ended June – July 2005. A total of 3874 subjects were part of the 1st year efficacy cohort.

Vaccine composition: (Table 25)

Vaccine	Formulation
GSK Biologicals' HRV vaccine	RIX4414 HRV strain derived from the 89-12 HRV vaccine strain 106.5 median Cell Culture Infective Dose (CCID50) Dulbecco's Modified Eagle Medium (DMEM) 3.7 mg Sucrose 9 mg Dextran 18 mg Sorbitol 13.5 mg Amino acids 9 mg
GSK Biologicals' diluent	Calcium carbonate 80 mg Xanthane 3.25 mg Water for injection q.s. ad 1.3 ml

15.2. Vaccine efficacy

The HRV vaccine was highly effective in protecting against RV GE during the first efficacy period. Vaccine efficacy was **87.1%** (95% CI: 79.6%; 92.1%) against any episodes of RV GE and **95.8%** (95% CI: 89.6%; 98.7%) against severe RV GE episodes. For increasing disease severity (Vesikari scores between 11 and 20), vaccine efficacy was increasingly higher, reaching 100% in a population having RV GE with a Vesikari

score ≥ 17 points. Vaccine efficacy against hospitalization for RV GE was **100%** (95% CI: 81.8%; 100%) and against RV GE episodes requiring medical attention was **91.8%** (95% CI: 84.0%; 96.3%) (Tables 26 and 27).

Table 26 - Percentage of subjects reporting any and severe RV GE, percentage of subjects hospitalized due to RV GE episodes, and vaccine efficacy during first efficacy period – cohort for efficacy

Groups		Any RV GE					Severe RV GE (score ≥11 on Vesikari scale)				
		Subjects			Vaccine efficacy		Subjects			Vaccine efficacy	
	N	n	%	P-value	% VE	95%CI	n	%	P-value	% VE	95%CI
$10^{6.5}$ $CCID_{50}$	2572	24	0.9	<0.001	**87.1**	79.6-92.1	5	0.2	<0.001	**95.8**	89.6-98.7
Placebo	1302	94	7.2	-	-	-	60	4.6	-	-	-

		Hospitalized RV GE				
		Subjects			Vaccine efficacy	
	N	n	%	P-value	% VE	95%CI
Study						
$10^{6.5}$ $CCID_{50}$	2572	0	0.0	<0.001	**100**	81.8-100
Placebo	1302	12	0.9	-	-	-

N = number of subjects included in each group
N% = number/percentage of subjects reporting at least one specified RV GE episode in each group
P-value = two-sided Fisher's exact test (significant level of α=0.05)
% VE = observed vaccine efficacy
95%CI = 95%Confidence Intervals
a score ≥11 on the 20-point Vesikari scale was defined as severe

Table 27 - Percentage of subjects reporting RV GE episodes requiring medical attention and vaccine efficacy during the first efficacy period – cohort for efficacy

Groups	N	RV GE requiring medical attention				
		n	%	Vaccine efficacy % VE	95% CI	*P-value*
$10^{6.5}$ $CCID_{50}$	2572	10	0.4	91.8	84.0-96.3	*<0.001*
Placebo	1302	62	4.8	-	-	-

N = number of subjects included in each group;

N/% = number/percentage of subjects reporting at least one RV GE episode requiring medical attention in each group;
P-value=two-sided Fisher's exact test (significant level of α=0.05);
% VE = observed vaccine efficacy, 95%CI = 95% Confidence Intervals

The HRV vaccine was highly protective against any and severe RV GE caused by G1P[8], G3P[8], G4P[8] and G9P[8] strains (Table 28).
Protection against G2P[4] RV type that does not share any of the outer capsid antigens of the HRV vaccine was lower in this study however the results of a meta analysis taking into account phase II and III efficacy studies showed a significant protective efficacy against any and severe GE due to G2P[4] (see Example 13).

Table 28 - Percentage of subjects reporting any or severe RV GE episodes and vaccine efficacy by serotype during the first efficacy period – cohort for efficacy

Groups		**Any RV GE**				**Severe RV GE (score ≥11 on Vesikari scale)**			
Viral Conc ***RV Strain***	N	n	%	Vaccine efficacy % VE 95% CI	*P-value*	n	%	Vaccine efficacy % VE 95% CI	*P-value*
G1 P[8] Wild type									
$10^{6.5}$ $CCID_{50}$	2572	4	0.2	**95.6** 87.9-98.8	<0.001	2	0.1	**96.4** 85.7-99.6	<0.001
Placebo	1302	46†	3.5			28†	2.2		
G2 P[4]									
$10^{6.5}$ $CCID_{50}$	2572	3	0.1	**62.0** -124.4-94.4	0.234	1	0.0	**74.7** -386.2-99.6	0.263
Placebo	1302	4	0.3			2	0.2		
G3 P[8]									
$10^{6.5}$ $CCID_{50}$	2572	1	0.0	**89.9** 9.5-99.8	0.018	0	0.0	**100** 44.8-100	0.004
Placebo	1302	5	0.4			5	0.4		
G4 P[8]									
$10^{6.5}$ $CCID_{50}$	2572	3	0.1	**88.3** 57.5-97.9	<0.001	0	0.0	**100** 64.9-100	<0.001
Placebo	1302	13†	1.0			7†	0.5		
G9 P[8]									
$10^{6.5}$ $CCID_{50}$	2572	13	0.5	**75.6** 51.1-88.5	<0.001	2	0.1	**94.7** 77.9-99.4	<0.001
Placebo	1302	27	2.1			19	1.5		
Pooled non G1 P[8] (G2, G3, G4, G9)									
$10^{6.5}$ $CCID_{50}$	2572	20	0.8	**79.3** 64.6-88.4	<0.001	3	0.1	**95.4** 85.3-99.1	<0.001
Placebo	1302	49	3.8			33	2.5		

N = number of subjects included in each group;
n/% = number/percentage of subjects reporting at least one RV GE episode in each group;
P-value = two-sided Fisher's exact test (significant level of α=0.05);

% VE = observed vaccine efficacy,
95%CI = 95% Confidence Intervals
a score ≥11 on the 20-point Vesikari scale was defined as severe
† One subject from the Placebo group counted in G1 and G4 categories since both serotypes were isolated

15.3. Summary of results

Two oral doses of HRV Rotarix™ vaccine, co-administered with childhood vaccinations, were highly effective during the first efficacy period compared to the placebo in protecting infants against any RV GE caused by G1P[8] wild-type RV and by non-G1P[8] RV types, vaccine efficacy was 95.6% (95% CI: 87.9%; 98.8%) and 79.3% (95% CI: 64.6%; 88.4%) respectively. The efficacy against severe RV GE caused by G1P[8] wild-type RVs and by non- G1P[8] RV types is 96.4% (95% CI: 85.7%; 99.6%) and 95.4% (95% CI: 85.3%; 99.1%) respectively.

These results are very supportive towards the conclusion that the HRV vaccine provides broad coverage against circulating RV strains (see Table 28: G1P[8], G2 P[4], G3P[8], G4P[8], G9P[8]). A meta analysis on vaccine efficacy against G2 P[4] specifically was performed, please refer to Example 13.

Overall Conclusions

The RIX4414 rotavirus vaccine proved to be highly protective against rotavirus gastroenteritis episodes measured by a clinical definition for case capture focusing on hospitalization and re-hydration, as well as by the validated Vesikari scale which includes quantifiable morbidity outcomes related with diarrhea, vomiting, fever, dehydration and hospitalization. Two oral doses of HRV vaccine were highly efficacious in protecting infants against any and severe RVGE and hospitalization due to multiple circulating rotavirus strains.

A high level of protection was demonstrated against homologous G1P[8] rotaviruses, which have two outer capsid proteins (VP4 and VP7) and one inner capsid protein (VP6) antigenically similar to the HRV vaccine. It also protected well against strains sharing only the genotype P[8] (VP4 antigen) and the VP6 antigen . Protection against rotavirus strains not sharing any of the outer capsid antigens of the HRV vaccine was also demonstrated in a meta analysis including the results of three phase II studies from Finland, Singapore and Latin America (all using identical methodology and efficacy

criteria) and of 2 phase III studies from Latin America and Europe, and which are reported in Example 13, vaccine efficacy against G2P[4] type of any severity was 81 % (95 % C.I. 31.6-95.8) and vaccine efficacy against severe GE due to G2P[4] type was 71.4 % (95 percent C.I. 20.1-91.1) indicating that the vaccine can also protect against strains which do not share identical G or P proteins with the vaccine strain.

Claims

1. A method of inducing an immune response against rotavirus strain, the method comprising administering to a subject a composition comprising an attenuated rotavirus strain of a GxPy type, said composition generating an immune response against a rotavirus strain which is neither a Gx nor a Py type.

2. Use of an attenuated rotavirus strain from GxPy type in the manufacture of a medicament for inducing an immune response against rotavirus infection caused by a rotavirus strain which is neither a Gx nor a Py type.

3. A method or use according to any preceding claim wherein the composition comprises a rotavirus having a VP4 gene comprising, in the nucleotide sequence, at least one of the following: an adenine base (A) at position 788, an adenine base (A) at position 802 and a thymine base (T) at position 501 from the start codon.

4. A method or use according to claim 3 in which the VP4 gene comprises a nucleotide sequence comprising an adenine base (A) at positions 788 and 802 and a thymine base (T) at position 501 from the start codon.

5. A method or use according to any of claims 1- 4 in which the composition comprises a rotavirus having a VP7 gene comprising, in the nucleotide sequence, at least one of the following: a thymine (T) at position 605, an adenine (A) at position 897 and a guanine (G) at position 897 from the start codon.

6. A method or use according to claim 5 in which the VP7 gene comprises a nucleotide sequence comprising a thymine (T) at position 605 and an adenine (A) or a guanine (G) at position 897 from the start codon.

7. A method or use according to any of claims 1 to 6, wherein the composition comprises a rotavirus having a VP4 gene comprising, in the nucleotide sequence, an adenine (A) at positions 788 and 802 and a thymine (T) at position 501 from the start codon; and wherein the VP7 gene comprises, in the nucleotide sequence, a thymine (T) at position 605 and an adenine (A) at position 897 from the start codon.

8. A method or use according to any of claims 1 to 7 wherein the composition comprises a G1 rotavirus strain and is used to induce an immune response to G1 and at least one of the non-G1 serotypes selected from the group consisting of: G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13 and G14 serotypes.

9. A method or use according to claim 8 wherein the composition comprises a G1 rotavirus strain and is used to induce an immune response to the G1 and G2 serotypes.

10. A method or use according to claim 9 wherein the composition is additionally able to induce an immune response to at least one of the non-G1 serotypes selected from the group consisting of: G3, G4 and G9.

11. A method or use according to any of claims 1 to 10 wherein the composition comprises a P[8] rotavirus strain and is used to induce an immune response to P[8] and at least one of the non-P[8] types selected from the group consisting of: P[1], P[2], P[3], P[4], P[5], P[6], P[7], P[9], P[11], P[12] , P[14] and P[19] types.

12. A method or use according to claim 11 wherein the composition is used to induce an immune response to the P[4] type.

13. A method or use according to any of claims 1 to 12 wherein the composition comprises a G1P[8] rotavirus strain and induces an immune response to a G2P[4] rotavirus strain.

14. A method or use according to any preceding claim wherein the composition comprising an attenuated rotavirus strain of a GxPy type provides protection against severe rotavirus-induced gastroenteritis caused by infection of a rotavirus strain which is neither a Gx nor a Py type .

15 A method or use according to any of claims 1 to 14 wherein the composition comprising an attenuated rotavirus strain of a GxPy type is at least 40% protective, in a population of vaccinated individuals, against diarrhoea caused by a rotavirus strain

which is neither a Gx nor a Py type of a rotavirus strain which is neither a Gx nor a Py type.

16. A method or use according to claim 15 wherein the composition is at least 50% protective.

17. A method or use according to any preceding claim wherein the composition is at least 60% protective.

18. A method or use according to any of claims 14 to 17 wherein the composition is between 40% and 80% protective.

19. A method or use according to claim 18 wherein the composition is between 50% and 70% protective.

20. A method or use according to any of claims 1 to 19 wherein the composition comprises a G1P[8] rotavirus strain which is between 40% and 75% protective in a population of vaccinated individuals against severe gastro-enteritis caused by infection of rotaviruses with a G2P4 serotype.

21. A method or use according to any of claims 1 to 20 wherein the rotavirus strain in said composition is ECACC deposit 99081301, or is obtainable or derivable from ECACC deposit 99081301.

22. A method or use according to any of claims 1 to 21 wherein the composition is administered in a 2-dose regime.

23. A method or use according to any of claims 1 to 21 wherein the attenuated rotavirus strain is formulated with a suitable pharmaceutical carrier or with an antacid buffer or both.