SCIENTIFIC DISCUSSION

This module reflects the initial scientific discussion for the approval of Dukoral. For information on changes after approval please refer to module 8.

1. Introduction

Dukoral is intended for active immunisation against cholera in adults and children from 2 years of age who will be visiting endemic/epidemic areas. The use of Dukoral should be determined on the basis of official recommendations taking into account the variability of epidemiology and the risk of contracting disease in different geographical areas and travelling conditions.

Dukoral has been licensed in Sweden since 1991 with chemically purified Cholera Toxin (CTB). A national variation for the introduction of Cholera Toxin produced by recombinant DNA technology (rCTB) was submitted and granted in Sweden in 1992. Dukoral was initially available in the Cholera indication only and received an extension of indication in 1995 in Sweden for traveller's diarrhea caused by LT-ETEC. With the effect of the Commission decision, the national authorisations are expected to be phased-out with an appropriate transition/communication plan to be provided by the applicant. The details of the plan shall be agreed nationally with the Member State concerned as reflected in the letter of commitment.

Due to the recombinant nature of one of the active ingredients, Dukoral qualifies as a part A product. The application was submitted in accordance with Art. 8.3. of the Commission Directive 2001/83/EC, as amended with studies carried out by the applicant and of bibliographical references (mixed application).

Cholera

Bacteriology

Epidemic and pandemic cholera disease is caused by *Vibrio cholerae*, mainly of serogroup O1 that produces cholera toxin. The division of *V. cholerae* into different O serogroups is based on its major surface antigen, the heat stable endotoxin. The endotoxin is a lipopolysaccharide (LPS), where the lipid portion of the molecule is embedded in the cell wall and the polysaccharide extends out from the surface of the bacteria.

Two dominating serotypes (subtypes) of serogroup O1, Ogawa and Inaba, have been identified. *V. cholerae* O1 strains have been shown to shift between these two serotypes. The O1 polysaccharide is built up from repeating units of a perosamine. The Ogawa polysaccharide has a single 2-*O*-methyl group in its terminal perosamine unit, which is absent in Inaba LPS. *V. cholerae* Inaba strains have been shown to be mutants of wild-type Ogawa strains that have lost the ability to methylate the terminal perosamine. *V. cholerae* O1 strains have been further divided into two biotypes, Classical and El Tor. El Tor strains differ from Classical strains by agglutinating chicken erythrocytes, mediated by the mannose sensitive haemagglutinogen (MSHA).

An antibacterial response is seen in humans after natural disease. The anti-bacterial response is measured as serum vibriocidal antibodies, i.e. the ability of a serum to kill *V. cholerae* bacteria in the presence of complement. This assay has been found to show the best correlation to protection. It has been shown that most of the vibriocidal antibodies are directed against LPS,. It is not believed that vibriocidal antibodies are directly involved in protection but their presence serves as a marker of intestinal secretory IgA antibodies against LPS, which mediate the actual protection.

Disease and epidemiology

Cholera continues to threaten many countries and constitutes a major global public health problem. In 1998, worldwide, a total of 293,111 cases of cholera and 10,586 deaths from cholera were reported to the World Health Organization (WHO). Since these figures are based only on official reports to the WHO, they probably underestimate the true numbers of cholera cases and deaths. Nevertheless, compared with previous years, the 1998 figures represent a significant increase in reported cholera cases and deaths worldwide, despite continued efforts to provide clean drinking water and basic sanitation.

Cholera is spread via contaminated water or food with humans as the only known host.. In endemic areas the prevalence is highest in children and decreases with age, as immunity is acquired. In non-endemic areas the cholera prevalence is not age-dependent, as all individuals are non-immune. Risk factors for cholera include low socio-economic status, poor sanitation, poverty, hypochlorhydria, lack of breastfeeding in infants and blood group O (for El Tor cholera). Typical symptoms of cholera are watery diarrhoea associated with dehydration. The fluid loss can be up to 20 liters a day, which leads to severe dehydration, unconsciousness and death within 18 hours if untreated. When an epidemic strikes an area where health care is not adequate the results can be disastrous, as happened in a refugee camp in Goma, Zaire in 1994. An estimated 58,000-80,000 cases and 23,800 deaths occurred within one month.

We are still experiencing the 7th cholera pandemic, caused by the El Tor biotype of *V. cholerae* O1. It started in 1961 in Indonesia and has since then spread across the world, reaching West Africa in 1970 and South America in 1991. By the end of 1996, cholera had spread to 21 countries in Latin America, causing over 1 million cases and more than 11,000 deaths. Until 1992 only *V. cholera* serogroup O1 caused epidemic cholera, but that year a previously unrecognised serogroup of the El Tor biotype, designated O139 (synonym Bengal), was discovered during large cholera outbreaks in India and Bangladesh. Isolation of *V. cholera* serogroup O139 has since been reported from 11 countries in South-East Asia The 0139 serogroup has been confined to this area without any tendency of pandemic spread so far. According to the WHO *V. cholerae* O139 accounted for 15% of laboratory-confirmed cholera cases in a cholera-endemic country of Asia. Cholera epidemiology in Calcutta, India 1992-98, has revealed that O139 dominated during 1992-93 and 1996-97, whereas O1 strains predominated during the rest of the period.*V. cholerae* strains falls into two groups based on serotyping; O1 and non-O1. The O1 strains are associated with epidemic outbreaks, whereas the non-O1 strains, with the exception of the above-mentioned O139, can cause sporadic cholera-like disease. Worldwide, *V. cholerae* O1, biotype El Tor predominates, whereas the Classical biotype exists in certain locations on the Indian subcontinent.

Vaccines and treatments

Conventionally, strategies to prevent cholera have focussed upon basic sanitary and hygiene measures such as treated water supplies, improving water delivery and sewage control, hand washing facilities, latrines and adequate hygiene in food handling. It is important to continue to support these recommendations because they are efficient when properly applied, but it is also recognized that they are often difficult to implement in full.

The treatment for cholera recommended by the WHO is oral rehydration solution (ORS), which reduces mortality from 50% down to 1%. In severe cases intravenous fluid replacement is necessary. Antibiotics can be used to treat severe cholera and may shorten the duration of disease and thereby to decrease the risk for further spread of the disease. Misguided use of antibiotics has led to the emergence of multiresistant cholera strains

The best way to avoid cholera is to have access to safe water supplies, and avoid contaminated foods. However, safe water supplies are not available to a great proportion of the world population. Therefore, a cholera vaccine has an important role in preventing illness and death in areas where good sanitation is difficult to implement. It should be clear that a cholera vaccine is considered only as an

additional tool to prevent cholera and will not replace any of the other cholera prevention and control interventions recommended by the WHO.

A parenteral vaccine based on inactivated *V. cholerae* O1 has been available for more than 40 years, but only offers up to 50% protection for 3-6 months and is associated with considerable local and systemic adverse reactions. WHO does not recommend this vaccine? Since both the cholera vibrios and the toxin they produce remain localised to the intestinal surface and lumen and exert their action locally on the epithelium during infection, local intestinal immunity is of critical importance for protection. The most efficient way of eliciting an intestinal IgA response is by oral vaccination. It is against this background that the development of oral rather than parenteral vaccines against cholera has been in the focus over the past 20 years. Dukoral was developed as an oral vaccine, containing both killed bacterial cells and the cholera toxin B subunit, thereby including the most important known protective antigens. Another oral cholera vaccine has also been licensed in certain countries it is a live attenuated vaccine based on the genetically manipulated *V. cholerae* strain CVD103-HgR. Although this vaccine had shown promising efficacy in challenged North American volunteers, it did not show adequate efficacy when tested in an endemic area and has therefore not received a general recommendation by WHO.

In a recent position paper (2001. Choleravaccines. WHO position paper: Weekly Epidemiological Record 76:117-124) of the WHO the following recommendation is given: "Among the new-generation cholera vaccines, convincing protection in field situations has been demonstrated only with the WC/rBS vaccine. Thus, the WC/rBS (Dukoral) vaccine should be considered in populations believed to be at imminent risk of a cholera epidemic." For immunization of travellers to highly endemic areas either of the two oral cholera vaccines could be used according to the WHO.

2. Chemical, pharmaceutical and biological aspects

Composition

The composition of the vaccine is given in the following table.

Table 1. Complete composition of Dukoral

Ingredient	Reference	Amount	Function
Recombinant CTB		1 mg	Active ingredient
Vibrio cholerae O1 Inaba classical biotype, heat inactivated		25x 109 bacteria	Active ingredient
Vibrio cholerae O1 Inaba El Tor biotype, formalin inactivated		25x 109 bacteria	Active ingredient
Vibrio cholerae O1 Ogawa classical biotype heat inactivated		25x 109 bacteria	Active ingredient
Vibrio cholerae O1 Ogawa classical biotype, formalin inactivated.		25x 109 bacteria	Active ingredient
Phosphate buffered Saline pH 7.2-7.4	Ph Eur	Ad 3 ml	Buffer

The Phosphate buffered Saline (PBS) consist/ ml of Sodium dihydrogen phosphate monohydrate 0.576 mg Disodium phosphate dihydrate 3.13 mg, Sodium chloride 8.5 mg and water for injections ad 1 ml.
The vaccine is filled in a colourless glass vial with a bromobutyl rubber stopper and a polypropylene screw cap. The buffer is supplied in paper sachets. Standard materials are being used for packaging material (glass vials, rubber stoppers, and screw caps) in compliance with the specifications and requirements of the European Pharmacopoeia.

A bicarbonate buffer is included in the product and is taken with the vaccine in order to neutralise the stomach acid that otherwise destroys CTB. Its nature has changed during development. The current formulation is in the form of an effervescent granulation dispensed into sachets and contains saccharin sodium as a sweetening agent and raspberry flavour as aroma. There are no functional differences, the main issue being the buffering capacity, between the various preparations. The production of the buffer is straightforward.

The buffer sachet contains the following:

Ingredient	Reference	Amount	Function
Sodium hydrogen carbonate	Ph Eur	3. 6 g	Effervescent agent
Citric acid anhydrous	Ph Eur	1,45 g	Effervescent agent
Raspberry flavour	Own monograph	70 mg	Aroma
Saccharin sodium	Ph Eur	30 mg	Sweetening agent
Sodium carbonate	Ph Eur	400 mg	Effervescent agent
Sodium citrate	Ph Eur	6 mg	Effervescent agent
Water, purified	Ph Eur	Disappears during manufacture	Granulating liquid

Active substance

The active substances are Recombinant Cholera toxin B subunit and four whole cell bulks; *Vibrio cholerae* O1 Inaba, classical biotype, heat inactivated, *Vibrio cholerae* O1 Inaba, El Tor biotype, formalin inactivated, *Vibrio cholerae* O1 Ogawa, classical biotype heat inactivated and *Vibrio cholerae* O1 Ogawa, classical biotype, formalin inactivated.

Whole cell bulks

Specifications for monovalent bacterial bulks

Test attribute	Test method	Specification
Physical appearance	Visual inspection	Beige opalescent suspension
pH	Potentiometry	6.7-7.6
Homogeneity	Visual	Homogeneous suspension. No visible aggregation
Purity	Gram staining	Only Gram negative rods
Identification of serotype	Slide agglutination	Agglutination with serotype specific antibodies
LPS concentration	Inhibition ELISA	Tested and reported
LPS concentration/ 10^{11} bacteria		> 400 EU/ 10^{11} bacteria for Inaba El Tor > 350 EU/ 10^{11} bacteria for heat inactivated Inaba, classical > 850 EU/ 10^{11} bacteria for Ogawa, classical heat and formalin inactivated.
Sterility	Ph Eur Direct inokulation	Sterile
Innocuity	Mouse weight gain assay	No decrease in weight, no signs of morbidity
Residual formaldehyde (when relevant)	Colour reaction	NMT 10 mM
Residual Cholera toxin activity	GM-1 ELISA	< 50 ng/ 10^{11} bacteria

The testing fulfils the WHO requirements.

The bacterial bulks are produced from established seed-lot systems that are controlled by standard microbiological and biochemical methods generally used in vaccine production. The microbiological methods include viability, purity (Gram-staining), and agglutination (Ogawa or Inaba serotype specific antibodies). The biochemical properties include fermentation pattern of sugars, haemagglutination, haemolytic activity, acetoin production, and polymyxine sensitivity. In all, the tests identify the serotype (Ogawa or Inaba) and the biotype (classical or El Tor) of cholera bacteria belonging to serogroup O1. The El Tor biotype differs from classical by displaying haemagglutinating and haemolytic activity, acetoin production, and polymyxine resistance.

The whole cell bulks are produced using standard techniques. The different seeds are grown in 550 L fermenters using a well-characterised cholera medium. The cells are harvested and concentrated. The concentrated suspension is then either subjected to heat inactivation at 56°C or formalin inactivation (0.5 %). The formalin bulks are then subjected to a 2nd concentration step to remove residual formaldehyde. The inactivated material is filled in Hyclone plastic bags and stored at 5°C.

Both heat- and formalin-inactivation processes have been appropriately validated. It is important to note that validation deals with the inactivation of the bacteria and not necessarily with the inactivation

of the toxin. However, data have been shown that the level of residual toxin is very low (not more than 20 ng per dose). A routine test for this has been introduced.

rCTB

Specifications for rCTB bulks

Test attribute	Test method	Specification
Physical appearance	Visual inspection	Clear, colourless to weakly yellow solution. Some particles may occur.
Identification	Ouchterlony immunoelectro-phoresis	Immunological identity with rCTB and CTB
pH	Potentiometry	7.0-7.6
Antigen concentration	Mancini	> 1 mg rCTB/ml
Protein content	Kjeldahl	>1 mg protein/ml
Antigenic purity	Antigen content/ protein content	NLT 0.8 mg rCTB/ mg protein
Purity	RP-HPLC	< 10 % unrelated proteins
Purity	SDS-PAGE	Not more than 2 bands visible; one major at 12 kD and one minor if present at 23 kD
Purity	SE-HPLC	Area of pentamer peak > 90 % of integrated area.
Sterility	Ph Eur Membrane filtration	Sterile

The recombinant CTB is a protein of 102 amino acids containing one disulphide bridge (cys 9- cys 86). It is produced in *Vibrio cholerae* strain 213 of serotype Inaba, biotype classical with a deleted CTA gene. A plasmid containing the CTB gene and an ampicillin resistance gene has been introduced. The seed lot system is tested for *V cholerae* characteristics as well as plasmid retention and ability to produce rCTB. In the preparation of seed lots and preculture, ampicillin is used in the culture medium while it is removed during the main fermentation.

During development of the production method for CTB, some changes have been introduced. The initially used native CTB has been replaced by a recombinantly produced CTB. Both molecules have been extensively characterised and shown to be comparable, except for six short peptide extensions at the N-terminal in rCTB. Several changes have also been introduced in the rCTB process, which increased the purity of the rCTB preparation, but did not alter the characteristics of the CTB protein. In former processes, plasmids were lost when not grown under ampicillin pressure. This is no longer seen in an improved method of preparation where a continuous feed of glucose and an addition of casamino acids are introduced.

The main fermentation is performed in 500-litre scale at +36 °C with aeration and agitation designed to keep pO_2 at 30 %. The fermentation is terminated after approximately 18 hours by cooling (OD at 600 nm shall be at least 8). The suspension is harvested and concentrated by ultrafiltration (MWCO 1000 kD). The concentrate is precipitated by addition of sodium hexametaphosphate (2g/L) and adjustment of pH to 4.9. The precipitate is stored at 2-8°C for 14 hours to 5 days. The solution containing the dissolved rCTB is centrifuged and filtered to remove non-dissolved matter (e.g LPS, other lipids and proteins. The rCTB is then purified by hydroxyapatite chromatography.

The collected rCTB peaks are pooled and the buffer is changed to a 0.02 M phosphate buffer. The rCTB solution is then membrane-filtered (0.22 um) and aseptically dispensed in 1 or 2 L borosilicate glass bottles. The rCTB production process has been validated by production and characterisation of three consecutive rCTB batches. Testing critical variables throughout the process showed process consistency. Characterisation of the three rCTB batches produced also demonstrated batch-to-batch consistency.

Characterisation

Data to show the characteristics of rCTB have been submitted including comparisons with native CTB (used in the initial clinical studies). The process for native CTB resulted in a very pure protein, as rigorous purification processes have to be applied for native CTB to remove all traces of Cholera Toxin A. For rCTB, expressed from a strain which cannot express the CTA and which should be blended with a mixture of killed whole cell bulks of the same bacterial specie, the absolute purity is of less importance. However, data from rCTB analyses of the current process indicates that the purity of the rCTB currently produced is comparable to the purity with the CTB used in the initial trials.

The characterisation is not as extensive as one normally sees for recombinant products but taken all together nevertheless deemed sufficient for its purpose. Further details can be found in the discussion part of this Report. Five or six molecular species with different molecular mass corresponding to different N-termini were found in MS analysis. The findings are consistent with what was found in N-terminal analysis where 6 different N-termini were detected, all of them extensions of the native CTB up to 7 amino acids. No truncated forms compared to native CTB were found. The results are due to the construct of the vector and expression cassette and the variants found were the expected, based on the construct and forms in between. After long time storage of the rCTB under refrigerated conditions and shorter under RT, there is a tendency of a shoulder of the peak found in the RP-HPLC which is explained to be related to the presence of low proteolytic enzyme activity, probably due to a Type I signal peptidase. In no case has a sequence shorter than the native CTB been seen and the Protease activity has decreased in later batches. In any case, the result of the trimming is a "more correct", i.e. less extended, form of the protein, hence the finding raises no concern. In view of the route of administration, the risk associated with the peptidase activity in the formulation is negligible when administered to humans and of no significance to the safety of the product

Active substance stability

Whole cell bulks

The applicant proposes a storage period of 3 years under refrigerated conditions for the whole cell bulks, which is substantiated by based on stability data. The tests of the bacterial bulks were physical appearance, pH, homogeneity, innocuity, O1-LPS content, and sterility.

rCTB

The tests of the rCTB bulks were physical appearance, sterility, pH, absorbance at 280 and 310 nm, protein nitrogen, SDS-PAGE, RP-HPLC, size exclusion chromatography, and antigen concentration as measured by single radial immunodiffusion (Mancini test).

Stability data for 36 months at 2-8 °C have been submitted both for material produced via the new process and using the former process justifying a storage period for 3 years at 5°C. In the intitial documentation the results shown for the new material all indicated a downward trend in antigenic content between 18 and 24 months. The manufacturer then submitted data from later time points from which it appears that the lower results at t=24 months was a single occasion event only and is not seen at later analyses.

A shoulder is seen in the main peak in RP-HPLC chromatograms of samples stored for 6 months at 25 °C. Upon even further storage, this shoulder may even turn into a distinct peak and could also be detected in samples stored at 5 °C. This phenomenon was not observed in samples stored at -70 °C. It was shown that this was the result of cleavage of the longer N-terminal extra amino acids present in rCTB into shorter extensions, due to the presence of minute amounts of the Signal peptidase I, responsible for cleavage of the signal peptide at the N-terminal of the rCTB molecule. However, no cleavage product shorter than native CTB has been found.

Other ingredients

The other ingredient of the preparation is a phosphate buffered saline which constituents fulfil the Ph Eur.

Product development and finished product

The composition of the product has been acceptably justified. The vaccine contains whole cell bacteria to create an anti-bacterial response and the B subunit of cholera toxin to create an anti-toxin response. The B subunit exists as a homopentamer surrounding the toxic A subunit (CTA). A response to the B subunit will also neutralise the toxic effect of the CTA.

The vaccine is formulated as an oral suspension. The antigens are aseptically mixed with autoclaved PBS and filled in 3.2 ml doses to allow for a extractable does of 3 ml.

The dosage form of the vaccine was chosen based on knowledge about intestinal immunity, indicating that that the oral route is superior to the parenteral for stimulating an immune response in the gut. The only additive used, PBS, was chosen to control the pH and salt concentration, in order to improve the stability of the antigens. Regardless of the fact that the vaccine is intended for oral administration it is formulated as a sterile product.

In the initial dose-finding clinical trials of the vaccine conducted by the University of Göteborg, the Swedish parenteral vaccine, albeit in a concentrated form, was used for establishing the oral dose of bacteria and CTB. One dose contained 25 x 10^9 of both heat-inactivated classical Ogawa and Inaba bacteria. In these trials, the vibriocidal response was found to be slightly lower than after natural disease and it was decided to double the dose of bacteria. As there were no bacteria of El Tor biotype in the vaccine it was decided to include an El Tor strain. Due to lack of knowledge of whether heat-inactivation might deteriorate protein antigens possibly contributing to protection, e.g. MSHA, a formalin-inactivation procedure was chosen for this strain. Since the El Tor strain selected was of serotype Inaba, it was for the same reasoning decided to include formalin-inactivated bacteria of serotype Ogawa. For simplicity, the classical Ogawa strain already included in the vaccine in a heat-inactivated form was chosen for this purpose. However, detailed information about the structure of the polysaccharide of O1 LPS was lacking at the time of the formulation of the vaccine. Accordingly, it could not be ruled out that formalin would have a negative effect on the immunogenicity of the LPS molecule. This state of knowledge laid the foundations for including *V. cholerae* O1 strains of both classical and El Tor biotypes and of both Ogawa and Inaba serotypes in the oral vaccine as well as using both heat- and formalin-inactivation of the two serotypes.

Furthermore, the vaccine was formulated with the aim of inducing both anti-bacterial and anti-toxic mucosal immunity in the gut after oral administration, a combination that had proven to protect synergistically in animal models. In order to achieve also anti-toxic immunity, the B-subunit of cholera toxin (CT) was included in the oral vaccine. Cholera toxin, like many other bacterial toxins, is built up from two subunits A and B. The A-subunit, itself built up by two peptides A_1 and A_2, exerts the toxic enzymatic activity of the toxin. The B-subunit, in the form of a homopentamer, binds the toxin to its target cell and delivers the A-subunit into the cell but has no toxic effect in itself. A majority of the antibodies against CT, obtained either after natural disease or immunisation, is directed against the B-subunit. Also, because most part of the A-subunit is embedded within the ring-formed homo-pentameric structure of the B-subunit, antibodies against the A subunit, even if they were induced, would have little effect on the neutralisation of the toxin. Using the B-subunit alone instead of the whole CT molecule in the oral cholera vaccine will thus not noticeably reduce the neutralising immune response.

For the manufacture, the monovalent cholera bulks are weighed with a nominal amount bacteria per dose of 25x 10^9. As the bacteria are inactivated, live count can for this reason not be performed. The amount added is instead added based on the bacterial content prior to inactivation.

The PBS is prepared and autoclaved at 121°C for 30 minutes. The buffer, the monovalent bulks and the rCTB bulk is aseptically mixed and stirred to homogeneity (3 hours). Following discussions with the applicant, a membrane filtration of the rCTB bulk has been introduced just prior to adding to the final bulk.Due to their nature the whole cell bulks can not be membrane filtered. Each vial is sealed with a rubber stopper and a screw cap with a safety ring.

The vaccine production process has been appropriately validated and is substantiated by batch analysis data, which conform with the pre-set criteria for acceptance including the final lot and final bulk specifications. Homogeneity of the bulk was maintained throughout the filling process, as demonstrated by measurements of the optical density at 600 nm of samples withdrawn during and after filling. Environmental monitoring and media fills also showed the aseptic nature of the process. Thus, the production process for the vaccine is shown to be suitable and to consistently yield a product of the desired quality.

Release and shelf-life specifications for Final bulk

Test attribute	Test method	Specification
Physical appearance	Visual control	Beige opalescent suspension
PH	Ph Eur	6.5 - 7.4
Homogeneity	Visual control	Homogeneous suspension, no visible aggregation
Purity	Gram staining	Only Gram negative rods
Antigen concentration		
O1-LPS	Inhibition ELISA	≥ 750 ELU/dose
rCTB	Mancini	0.8 - 1.2 mg/dose
Residual formaldehyde		< 6.7 mM
Sterility	Ph Eur Direct inoculation	No microbial growth
Identity	Slide agglutination	Agglutination with Inaba and Ogawa specific antibodies
	Ouchterlony	Identity with B subunit (rCTB/ CTB)

Table 5. Release and shelf-life specifications for Final lot

Test attribute	Test method	Specification
Sterility	Ph Eur Direct inoculation	No microbial growth
Identity	Slide agglutination	Agglutination with Inaba and Ogawa specific antibodies
	Ouchterlony	Identity with B subunit (rCTB/ CTB)

Withdraw able volume is not tested in the final specification. However, this is tested in process during filling.

Table 6. Release and shelf-life specifications for Dukoral finished product

Test attribute	Test method	Specification
Identity	Slide agglutination	Agglutination with Inaba and Ogawa specific antibodies
Control of labelling	Visual inspection	Labelling in accordance with specifications

A test for immunogenicity is also proposed but in line with the WHO discussions where it was found that this method had little or no relevance for determining the activity of an oral vaccine it is proposed that this is deleted.

Analytical methods have been appropriately validated and a good reproducibility is indicated by batch analysis data.

Biological starting materials are appropriately treated to kill viral contaminants that possibly could be present in the material. The seeds and the biological starting material are not considered as a source of viral contamination.

TSE risk assessment
Few components of animal origin are used in the production of the vaccine. Most of the material is either from species where no TSE issue exists (pig, horse) or from milk of bovine origin where the process is such that it falls out of the TSE guideline. A policy on which countries of origin are accepted is submitted.

Sodium taurocholate was used in the establishment of the seed lot system in 1996/97. No certification can be obtained from historical material. The material contains ovine bile of New Zealand origin. The taurocholate agar is used for passaging the seeds, which are then scraped off the agar plates. The agar is hard and great care is taken to remove only the bacteria in the preparation, so only very minute amounts, if any, of the agar will be transferred to the Working seed. From this, together with the geographical origin of the material (New Zealand) and the classification of bile as being of no detectable infectivity it is deemed that the TSE risk associated with the use of ovine bile is negligible and that the requirements can be considered as fulfilled. When producing new working seeds, the applicant should preferably use certified material and will report on future plans and progress on the introduction of such material.

Stability of the Product

Stability of the vaccine as well as of the buffer system as reflected by the shelf life in the SPC has been appropriately investigated and substantiated by batch analysis data with results remaining within the set specifications.

Discussion on chemical, pharmaceutical and biological aspects

Maufacturing, testing and stability of Dukoral are acceptably described. The product fulfills the WHO requirements as adopted in 2001.

Toxin Coregulated Pili (TCP) and mannose sensitive hemagglutinin (MSHA) have been suggested as influencing factors in the establishment of immunity to cholera. These factors are however not monitored in the production of Dukoral, thus their possible presence cannot be established. The role of TCP and MSHA in colonisation and pathogenesis in human cholera as well as immune response against TCP and MSHA and *in vitro* expression of TCP and MSHA has been investigated in several studies. Evidence has been provided to show that a) the amount of the antigens are likely very low in the product and b) they are not important for an inactivated vaccine like this justifying the absence of their monitoring.

The WHO requirements also require applicants to assure freedom from Zonula occludens toxin (ZOT) and Accessory cholera enterotoxin (ACE). Although no studies to verify this have been submitted, it is well known according to literature that strains deleted in the ZOT gene were more reactive compared with other strains containing the gene. Long experience of safe use exists for this vaccine and it is deemed that no further information on this is needed.

The mouse weight-gain assay, as applied by the applicant in the testing of the cholera whole cell bulks, is aimed at indicating general toxicity as extra precaution in addition to the abnormal toxicity test according to PhEur. This is due to the lack of a meaningful animal model for toxicity testing of oral killed whole cell cholera vaccine. The assay has however not been validated for the purpose of detecting residual cholera toxin in the routine testing of the bulks or at the finished product stage which is required according to the WHO requirements. Therefore, the applicant has agreed to introduce a properly validated GM1 ELISA for routine testing of bulks for the detection of residual cholera toxin. This introduction may make the mouse weight gain test superfluous, however, elimination of the test should be handled as a variation.

The characterisation of rCTB is not as extensive as one normally sees for recombinant products. It can be noted that there are no data from sequencing of the entire protein, only N and C-terminal sequences have been analysed. The protein carries one disulfide bridge and the correctness of the S-S bridge has not been shown as such by peptide mapping or by other methods. Methods to verify 2°structure like CD have not been used. On the other hand, powerful methods like MS have been used to determine the molecular weight and functional tests (GM1 binding, binding to monoclonal antibodies) have been performed. The DNA sequence has been confirmed and it is deemed unlikely that a protein emanating from the correct DNA sequence, with the molecular weight expected from the N- and C-terminal analyses and reacting comparably with native CTB in functional assays would differ from the expected structure.

Normally, SDS-PAGE data using a more sensitive staining such as silver staining would be requested. However, due to the fact that the rCTB is mixed with crude whole cell bulks, the absolute absence of contaminating, non-CTB proteins, which could be detected by silver staining, is of less importance. What is of importance is the integrity of the rCTB and this is better seen in the western blot analysis performed.

It should be noted that it is not possible to quantify the respective amount of Ogawa and Inaba LPS due to the lack of appropriate methods. Therefore, it is not possible to ensure that the proportions of Ogawa and Inaba LPS in the vaccine are correct. Notably, even if methods for quantifying the respective LPS were available, it would still not be possible to differentiate between LPS from heat- and formalin-inactivated bacteria, nor would it be possible to differentiate between LPS from classical and El Tor biotype. So, even if methods were available to measure Ogawa and Inaba O1 LPS separately, the designed content of the vaccine could still not be verified. In practice, however, working according to GMP guarantees the correctness of the composition of the vaccine.

In the same way, it is only possible to express the amount of bacteria in the final vaccine as the amount prior to inactivation. Although this is not optimal, it is the only other choice is to give the sum LPS content where it is not possible to differ between the contributions from each strain. Therefore, the current way of expression is deemed acceptable.

3. Toxico-pharmacological aspects

Introduction

The pre-clinical part of the application for Dukoral is composed of bibliographical references and studies carried out by the applicant. The content of the application has been assessed in line with the Annex I to Directive 2001/83 and the Note for Guidance on preclinical and toxicological testing for vaccines, the available literature data and the existing long-term clinical experience.

Pharmacology

The active components of the vaccine are recombinant cholera toxin subunit B (in 1992 production was changed to recombinant CTB; rCTB) and whole, heat-inactivated or formalin-inactivated, bacterial organisms. The B subunit (CTB) is the binding portion of the cholera toxin and is non-toxic by itself. The cellular receptor for CTB is GM-1 ganglioside, which is expressed on most mammalian intestinal cells. Antibodies against the toxin can prevent diarrhoeal disease that would otherwise follow. These antibodies are mostly directed against the B subunit but by combining the B subunit with inactivated whole cells of the bacteria the synergistic action of IgA antibodies against both the toxin and the cells can be obtained locally in the gut (Svennerholm AM and Holmgren J Infect Immun 1976. 13:735-40).

The recombinant CTB differs from native CTB in that the former contains up to 7 extra amino acids at the N-terminal. This has no effect on binding to the GM-1 receptor or to monoclonal antibodies as demonstrated in studies performed by the applicant.

There is no valid animal model to predict the mucosal immune response to the cholera vaccine (Richardsson SH in *Vibrio Cholerae and Cholerae Molecular to global perspective*. 1994.Chapter 14 American Society for Microbiology) this is because *V. cholerae* is a strictly human pathogen which excludes relevant animal challenge models. Further, killed Gram-negative bacteria are not immunogenic by the oral route in animals (not even in monkeys). However, some animal studies, especially of the mechanism of immune response and protection regarding the antitoxin immunity, have been conducted. Literature data indicate that most antibodies induced by immunisation with CT (cholera holotoxin) are directed against the atoxic B subunit (CTB) (Lange S and Holmgren J. Acta Path Mcrobiol Scan Sect C 1978, 86:145-152), and consequently the subunit cholera toxoid consisting of the purified CTB has been prepared in order to eliminate the risk of toxicity (Holmgren et al

Nature; 1977: 269:602-04,). However, as mentioned above CTB gives a poor mucosal antitoxin antibody response in mice after oral immunisation (Lycke et al. In Arch Allergy Appl Immunol 1989;88:273-79). This is thus in contrast to what has been found in humans.

Moreover, in the final draft of the WHO Guidelines for the Production & Control of Inactivated Oral Cholera Vaccines (WHO Techn Rep Ser. www.who.int/biologicals/Index/Cholera.htm) it is stated that: "At present no animal potency or immunogenicity assay can be recommended that can meaningfully be used as a reliable indicator of the protective efficacy of inactivated oral cholera vaccines in humans nor be able to detect sub-potent batches".

The NfG (CPMP/SWP465/05) states that the pharmacodynamic studies "should be carried out" or "considered" in "appropriate animal models". However, due to the lack of an appropriate animal model, the pharmacological part does not comprehensively describe the different aspects normally required for this part of the dossier.

Concerning safety pharmacology ("potentially undesirable pharmacodynamic effects") that is mentioned in the NfG it is stated that this should be considered for new vaccines. In the present case the issue has to rely on the considerable clinical experience.

Pharmacokinetics

In the relevant CPMP guideline (CPMP/SWP465/05) it is clear that pharmacokinetic studies are "normally not needed". This should be especially true for Dukoral, since the vaccine is taken orally and the components of the vaccine are not systemically absorbed from the intestine. One of the important physiological functions of the epithelium is to prevent bacteria from entering the underlying tissue and there are several theoretical reasons for not performing studies on kinetics. Some publications in the literature adequately discuss matters related to receptor binding capacity of proteins, and the specifics of antibody response after oral exposure vs parenteral (Aizpurua HJ, Rusel Jones GJ. J Exp Med 167; 440-51; Owen RL et al, J Inf Dis 53:1108-18, 1986; Quiding et al J Clin Invest 88:143-8, 1991).

Toxicology

- **Single dose toxicity**

Due to the lack of relevant animal models an extensive evaluation of the preclinical toxicity profile of this vaccine has not been conducted.

Considering the individual active components of the vaccine, rCTB (a protein of 102 amino acids) and killed whole cell bacteria, the species/tissue specificity suggest that standard toxicity tests would not be relevant. Oral intake of killed bacteria and protein is unlikely to cause any toxicity. It can be argued that due to the similarities that exist between common Gram-negative gut flora and killed cholera bacteria, specific toxic effects are not likely/can be excluded from occurring. Also, from the publication by Holmgren et al 1977 (mentioned above) it is clear that the properties of the subunit CTB in the vaccine are such that reversion to toxicity is prevented. This was demonstrated in rabbits that were administered 10 microgram of the subunit toxoid subcutaneously.

The active ingredients in the vaccine are thus unlikely to exhibit any inherent toxicity, but studies may also be of interest from the "quality" point of view, i.e. to test for potential toxicity of residues, impurities or any decomposition products. Animal data relating to the quality of the vaccine are available. These "abnormal toxicity tests" were conducted after intraperitoneal administration in mice and guinea pigs in compliance with the requirements described in the European Pharmacopoeia (Ph Eur 2.6.9,1997) and included clinical observations and gross pathology with up to 10 days observational periods. The intraperitoneal route is more likely to result in systemic absorption than the oral administration route. Forty-six vaccine batches have been tested since 1993 and have passed the test. Similar tests were performed in rabbits (102 cholera bulks and 160 vaccines). Non-toxicity of Dukoral was reported.

The CPMP/SWP/465/95 guideline on vaccines states that: "Single dose toxicity data from at least one species should be available". The particular nature of the Dukoral vaccine implies that standard toxicity tests would not be relevant as outlined above. Further, although not including histopathology data, "abnormal toxicity" testing and studies on immunogenicity and protection that indicate no adverse clinical reactions, no adverse gross pathology or effects on normal weight gain, are consistent with the non-toxicity of the vaccine. Additionally, in studies in man (Castello-Branco et al Vaccine 12:65-72, 1994; Clemens JD et al J Inf Dis 154:175-8 1986; Clemes JD et al Lancet 335:270-3, 1990), safety was actively studied as adverse events after a single dose of vaccine.

- **Repeated dose toxicity**

A study on repeated dose toxicity aimed at identifying target organs of toxicity is normally required as per the relevant NfG. There is a problem of lacking relevant animal models for preclinical studies with this vaccine more generally. Importantly though, extensive clinical experience indicates that additional animal studies would not contribute to the further understanding of the product's mode of action or safety profile.
In addition, immunological aspects of toxicity should be considered on a case-by -case basis. There have been no reports on auto-immunity being induced by V. cholerae (Medline search and clinical experience). As the cholera antigens in the vaccine do not include CT, the killed cells are not expected to behave differently from the common Gram-negative flora present in the intestine. Hypersensitivity is rather specific for the animal species and it was not detected in clinical trials analysed to date. Further, it should be noted that the cholera toxin B subunit is used in animal models to *induce* immunologic tolerance. Examples of such models are allergic encephalomyelitis, autoimmune diabetes and collagen-induced arthritis. This experience is summarised in a review by Holmgren et al (Holmgren et al Expert Rev Vaccines 2:205-17, 2003). This implies that CTB is not a potent inducer of hypersensitivity, but rather the opposite.

- **Genotoxicity in vitro and in vivo and Carcinogenecity**

Genotoxicity and carcinogenicity studies are normally not requested for vaccines. This is in line with the CPMP guideline (CPMP/SWP465/05). Formaldehyde, used in inactivation of bacteria, is carcinogenic at high doses; however, the residual amount of formalin in Dukoral is far below the level of risk and in compliance with the Ph. Eur. Requirements (≤ 0.2 g/L = 6.7 mM).

- **Reproductive and developmental studies**

It can be argued that given that the vaccine components are not absorbed, no effects on reproductive functions or embryos of pregnant women are expected. Based on literature/epidemiological data in pregnant women, (Freda, V Am J Obst Gynec 71: 1134 –36, 1956), cholera infection has not been described to affect the reproductive function or malformations of embryos, nor has any such possible relationship been reported or even suspected after vaccination with Dukoral or any other cholera vaccine. More than 1000,000 doses have been sold in Scandinavia and there are no ADR reports related to pregnancy or post partum conditions.

Part of the female population living in cholera endemic countries is likely to become infected with cholera when pregnant. Furthermore, in clinical studies conducted before the vaccine was registered, women were for research ethical reasons asked not to participate in the study if they would be pregnant during the study period. It could be assumed that an unknown number of pregnant women came to be included. Although an individual follow-up of these women was not done on a regular basis, there were no reports of pregnancy related complications or complications in the newborns that were linked to the intake of the vaccine.

Dukoral has been given to a large number of breast-feeding women in different studies, and no adverse events in relation to breast-feeding have been reported (Holmgren et al. Expert Rev Vaccines 2:205-17, 2003; Hirchhorn N. et al Lancet, 1: 1230-2, 1969).

The relevant CPMP guideline states: “embryo/fetal and perinatal toxicity are usually not necessary. Only if a vaccine is intended for use in women of child-bearing age or during pregnancy may such studies become necessary”.
Theoretical considerations are in line with that the product is unlikely to have any effects during pregnancy and pregnant women have been exposed to the vaccine. Furthermore, considering the inherent characteristics and properties of the product the relevance of preclinical data is questioned. It is clear that these aspects, as well as the overall safety of the vaccine, have to be judged on clinical data, alone. Cholera infection *per se* does not seem to adversely affect reproduction and this can be expected to be true for the components of the vaccine as well.

- **Local tolerance**

This being an oral vaccine, the only/most relevant way to assess toxicity is by administering it orally. As in the case with normal non-pathogenic live or dead bacteria, healthy animals are not expected to absorb the inactivated cholera bacteria or to respond immunologically. Also rCTB has been described as not being absorbed after oral administration**.** It reacts with the GM1-receptor of the epithelial cells in the intestine but remains locally.

In view of this and the vast experience of the vaccine in humans, studies of local gastrointestinal tolerance in animals are not warranted.

Environmental Risk Assessment

Considering the nature of the product (killed bacteria, no preservatives etc.) and the way it is manufactured, one does not expect any risks for the environment. Indeed, the two production sites of SBL Vaccin AB in Sweden, do fulfil the requirements from the Swedish local authorities for environment control.

Discussion on the non-clinical aspects

The applicant has discussed the extent of testing in relation to applicable guidelines and overall provided acceptable justification for not conducting preclinical studies. A summary report indicates that no regular GLP studies on the pharmacology and toxicology of the vaccine have been conducted. Since this concerns *V. Cholerae,* a strictly human pathogen, and a killed whole-cell vaccine, to be administered orally, the relevance of animal pharmacology studies is questioned. The natural disease apparently cannot be reproduced in adult animals and responses to CT and CTB differ between humans and animals. Specifically, CTB is not an effective oral immunogen in mice, but is so in humans. Overall, the mode of action is fairly well understood and efficacy has been shown in the clinic.

Concerning potential for toxicity, the individual active constituents of the vaccine; a protein of 102 amino acids and killed whole cell bacteria, imply that standard animal toxicity tests would not be meaningful. Further, there are studies dealing with immunogenicity and protection that are consistent with the non-toxicity of the vaccine**.** A study on repeated dose toxicity aimed to identify target organs of toxicity is normally required. However, inherent characteristics of the vaccine and the extensive clinical experience available indicate that additional animal studies would not contribute to a further understanding of the product’s mode of action and safety profile. The cholera vaccine has been available in Sweden since 1991 and over 1,000,000 doses have been distributed in Scandinavia to date. The clinical safety data include data from about 7,000 children, mostly from clinical trials in non-European countries. The adverse events reported include gastrointestinal reactions, likely due to the buffer used, fever, headache, dizziness and skin reactions. The vaccine is also intended for women of child-bearing potential. Theoretical considerations are in line with that the product is unlikely to have any effects during pregnancy and pregnant women have been exposed to the vaccine. Genotoxicity and carcinogenicity studies are not required for this type of product. An "Environmental risk assessment" is available and no risk is expected.

Preclinical aspects in relation to the ETEC indication are not specifically discussed in the marketing authorisation application. References to the published literature (REFS) indicate that the enterotoxin of *Escherichia coli* has similar immunomodulatory properties as cholera toxin

Dukoral containing the B-subunit of cholera toxin (CTB) has been on the market in Sweden since 1991 and since 1992 containing the recombinant B-subunit (rCTB). The recombinant CTB differs from native CTB in that the former contains up to 7 extra amino acids at the N-terminal. This has no effect on binding to the GM-1 receptor or to monoclonal antibodies. During purification of rCTB, hexametaphosphate and the anti-foam Adekanol LG-109 are used during fermentation, the former is accepted as a food additive and levels of the Adekanol are below detection levels.
There are acceptable justifications for not carrying out comprehensive non-clinical investigations for this vaccine. Some of these justifiifications are also sufficiently supported by the relevant CPMP guideline on preclinical pharmacological and toxicological testing of vaccines (CPMP/SWP/465/05).

From a scientific point of view, as discussed above, the paucity of preclinical studies to characterise the pharmaco/toxicological profile of Dukoral is not considered a cause for concern.

Overall, based on the particular characteristics of the vaccine and the clinical experience to date, additional preclinical studies are not warranted. The text in SPC sections 4.6 and 5.3 is appropriate and accurately reflects the lack of non-clinical data. Efficacy and the overall safety of the vaccine should be judged on clinical data.

In addition recital 10, of Annex I of Directive 2001/83/EC, as amended states: " However, there are reasons of public policy for not conducting repetitive tests on humans or animals without over-riding cause". Reference is also made to Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes, and Council Decision 1999/575/EC of 23 March 1998 concerning the conclusion by the Community of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

4. Clinical aspects

Introduction

The clinical part of the application is composed of studies carried out by the applicant and of bibliographical references. Many of those studies were conducted without the sponsorship of the company. For study 6, the Bangladesh efficacy study, the World Health Organization (WHO), the United States Agency for International Development (USAID), the Government of Japan and the Swedish Agency for Research Cooperation with Developing Countries (SAREC) provided financial support for the study. The Control of Diarrheal Disease Programme of the WHO monitored the study. The US Army performed study 27, the cholera efficacy study in Peruvian military personnel. The study was monitored by the USAMMDA's (US Army Medical Material Development Activity) quality assurance group.

The applicant has undertaken a reanalysis of data and produced clinical study reports for the key efficacy studies (Studies 6, 9 and 27). Other studies in this report were identified from literature searches and were investigator driven rather than sponsored by the applicant. Not all studies have been fully reported. The majority of the studies have been published in high-impact peer-reviewed scientific journals and have been reviewed for the submission document. For all studies, the sponsors followed local requirements with regard to approval by ethics committees and regulatory authorities. Since the studies were performed before the ICH guideline came into operation, they did not conform to the current ICH guideline on GCP.

Altogether 48 referenced clinical studies were submitted (see further "table of all clinical trials with Dukoral"), including 19 phase I (n=705), 20 phase II (n=4,951), 8 phase III trials (n=221,146) and in addition, one feasibility study (n=27,607). There were 19 placebo-controlled studies and 29 open

trials. Five unpublished studies were only provided as summaries. Three efficacy studies including 84,462 individuals were not successful and, thus, 136,664 study subjects were assessed in completed phase III efficacy trials. The clinical trial programme has run during the period of 1980 to 1997. The study populations include those from endemic and non-endemic regions, and also special populations such as children >2 years of age, HIV-infected individuals, patients with ulcerative colitis and those with IgA deficiency. A total of 25 clinical studies involved European subjects, whereof the majority was small Phase I and II trials assessing the immunogenicity and the safety of the vaccine.

Studies 11 and 15-48 (see table of all clinical trials with Dukoral) have been carried out with the recombinant vaccin.

Immunogenicity of the cholera vaccine was investigated in clinical studies and in several academic trials. Systemic and local intestinal immune responses to the vaccine were assessed as well as antibody responses in saliva, faecal samples and breast milk. Antibody responses of IgG and IgA classes to cholera toxin subunit B (anti-CTB) and to bacterial antigens, lipopolysaccharide (anti-LPS), and vibriocidal antibodies were measured. Antibody secreting cells (ASC) in blood and intestinal fluids were also measured. The immunogenicity studies aimed to establish vaccination schedules and booster intervals, and to evaluate a protective correlate and immunological memory.

As discussed in the non-clinical part there is no reason to require pharmacokinetic studies with this oral killed vaccine.

The efficacy of Dukoral against cholera was investigated in 3 randomised placebo-controlled studies (table 1). The cholera studies involved a total of 113,083 subjects of whom 30,812 received the assigned number of Dukoral (WC/BS vaccine) doses. A total of 13,760 children aged 2 to 5 years were recruited of whom 4,919 received the assigned number of WC/BS vaccine doses. The study populations for cholera included those from endemic areas (Bangladesh) and those from epidemic regions (Peru). Thus, for the cholera indication no formal efficacy studies have been performed, in the target group, i.e. naive travellers.

Table 1 Summary of study design of key efficacy studies

Study location (Number)	**Year**	**Dosage regimen**	**Number (Age groups)**	**Follow up**
		Cholera		
Bangladesh (Study 6)	1985-88	3 doses at 6 week intervals	**89,152** (2-65 years)	6 months-5 years
Peru, military (Study 27)	1994	2 doses 7-11 days apart	**1,563** (18-65 years)	5 months
Pampas (Study 30)	1993-95	2 doses 2 weeks apart with a booster dose 1 year later	**21,924** (2-65 years)	2 years

The whole-cell vaccine (WC/BS) originally contained cholera toxin B subunit (CTB) purified from cholera toxin produced by a wild-type strain. This vaccine, containing the native form of CTB, was used in the Bangladesh field efficacy trial (study 6) and in the Morocco trial (study 9). In 1991 production was switched to a recombinant form rCTB. The vaccine (WC/rBS) containing rCTB was used in the Peru military trial (study 27) and in the Pampas field trial (study 30).

Immunogenicity/vaccination schedules

Dose determination/vaccine composition

No formal dose determination studies were performed. The dose of CTB, 1mg, was determined on the basis of one early study in Bangladeshi volunteers, in whom immune responses to clinical cholera were compared with those after different oral doses of CTB (0.5 mg and 2.5 mg) plus WC. The native CTB was demonstrated to induce similar immune responses as rCTB (studies 11 and 17 and Sanchez J and Holmgren J. Proc Natl Acad Sci USA 86:481-5, 1989). A sodium bicarbonate buffer in full strength was shown to be necessary for protection of the acid labile CTB component and to retain immunogenicity of the vaccine, despite causing some gastrointestinal symptoms. The total bacterial content was increased to a total amount of $1x10^{11}$ vibrios/vaccine dose by adding an El Tor strain and a formalin inactivation procedure was introduced, due to the poor antibacterial responses achieved in non-immune individuals (Study 3). Since the protective efficacy (PE) of Dukoral in study 6 was deemed acceptable, no further change in the vaccine composition was made during the clinical development programme. It was discussed whether the present bacterial content by cholera biotype is optimal, since only one-fourth of the whole cells constitute El Tor strains, globally the predominant biotype. According to the Company the most important determinant of PE is the LPS component, which is common for the Classical and El Tor strains, but different for the serotypes. Therefore the most important goal is to obtain an even distribution of the Inaba and Ogawa serotypes, which prevails in the present vaccine. Since the vaccine has proven satisfactorily efficacious against both Classical and El Tor cholera (study 6 and 27), this issue was considered resolved.

Immunogenicity

The vaccine was demonstrated to be immunogenic in both children and adults and in individuals from endemic as well as non-endemic areas. A limited number of elderly subjects >65 years were included in the clinical trial programme, serological responses were consistent with those of younger individuals.. In the smaller trials significant antitoxin and antibacterial antibody responses were documented in serum, intestinal lavage fluid and other secretions, but the magnitude of antibacterial antibody responses in the serum and antitoxin antibody responses in the intestine were lower in non-primed individuals. In the larger serological studies, serum IgG and IgA antitoxin responses were induced in 70-90% of the vaccines. In contrast, very modest serum antibacterial antibody responses (17-45%) were noted both in endemic and non-endemic populations. The vibriocidal antibody responses were even more reduced in children and in individuals with high baseline titres. The poor vibriocidal response elicited by the WC/rBS vaccine raised some concern, since vibriocidal antibodies have been considered as reliable surrogate markers of protection against cholera. However, the data from the Bangladesh field trial indicated that despite low antibacterial titer levels a significant protective vaccine efficacy against cholera was obtained. In this field trial, significant antitoxin IgG responses (>2-fold increase) were observed in 73% of subjects, whereas few (17-21%) exhibited significant (>4-fold) vibriocidal antibody responses. Due to the limited number of cholera cases in the Bangladeshi subjects with serological samples collected, it was not possible to investigate any correlation between antibody levels and protection against cholera. In a separate sub-study no correlation was found between serological markers and vaccine efficacy. It was concluded that vaccine-induced mucosal immunity might be dissociated from vaccine-induced serological titres. In response to the issues raised over the poor vibriocidal response, it was clarified that the vibriocidal responses do not correlate directly to protective immunity after oral or parenteral vaccination, nor is there a direct correlation between vibriocidal response and clinical outcome of infection. Vibriocidal antibody levels in an endemic population increase with age, but cannot be used to diagnose infection or predict the immune status of an individual. In conclusion, vibriocidal antibody response in immunized subjects could be used as a surrogate marker of vaccination, but is not a good predictor of protective efficacy.

Vaccination schedules

- **Primary immunisation schedule**

No study was designed to specifically determine the optimal number of doses for primary immunisation. The recommended 2-dose schedule in adults and 3-dose schedule in children in the cholera indication were mainly based on efficacy results obtained in the Bangladesh field trial (Study 6), but were also supported by immunogenicity data in primed and non-primed populations. Dose intervals of at least one week to 6 weeks were selected based on 2 immunogenicity trials (one in children) and were supported by efficacy results. However, no data were submitted to justify the recommendation to restart the primary vaccination course if more than 6 weeks have elapsed between doses. The peak serum, intestinal antibody responses and ASC responses following vaccination were seen after on average 7 days. The early kinetics of the antibody response supports the recommendation for travelers to complete dosing at least 1 week before departure.
For cholera, immunogenicity data showed that 2 and 3 doses were as efficient in inducing both primary and memory antitoxin/antibacterial serum responses in naive adult volunteers. From an efficacy point of view, the 2-dose schedule in adults is satisfactorily documented primarily in an endemic Bangladeshi population. Data on the serum antibody responses in non-primed populations support the same schedule. No study of vaccine protection against cholera has been performed in the target population, i.e. non-immune EU travellers. The potential comparability between younger children in Bangladesh and healthy EU adults was discussed, but was not considered appropriate due to that several endemic factors, such as differences in nutritional status and gut flora, could affect the immune response to vaccination. Although no serological data were collected in the Peru military study, the high attack rate of cholera suggested that the recruits were non-immune, and thus would constitute a population most resembling that of naïve travellers. The PE of the vaccine in this trial was 85%. It was also discussed whether pre-exposure to LT-ETEC could afford some cross-immunity against cholera in the Peruvian population. However, no differences in anti-toxic antibody responses to CTB in serum or intestine could be demonstrated between study populations from endemic and non-endemic countries. The data did not indicate that previous exposure to ETEC and possible immune memory against LT prior to immunisation would enhance vaccine responses. Therefore, it was considered that efficacy data could be used to bridge from Bangladesh and Peru to a naive population of EU travelers.

The efficacy results obtained in Pampas field trial (Study 30) suggest that a 3-dose primary regimen may be needed for protection against El Tor cholera in a non/semi-primed population. However, the administrative problems (se below) associated with Study 30 make these results less reliable.
In Bangladeshi children (2-5 years) it was shown that the proportion of vaccine recipients exhibiting significant rise of antibody titres increased after each successive dose up to the 3rd dose supporting the use of a 3-dose regimen. Protective vaccine efficacy in children has only been documented for the 3-dose schedule.

- **Booster dose**

The recommendation of the timepoint for a booster dose of WC/rBS vaccine was based upon immunological correlates and data on duration of protection. Booster doses after 10-12 months following primary immunisation with 2 doses two weeks apart in Swedish and Latin American subjects were demonstrated to elicit anamnestic antitoxin and vibriocidal antibody responses. However, an elevated vibriocidal antibody titre at baseline resulted in a diminished booster response. In a small trial it was also shown that a booster dose administered after 5 months resulted in anamnestic antitoxin antibody and ASC responses locally in the intestine, suggesting the existence of a local immunological memory. A systemic immune memory was demonstrated for up to 5 years in subjects living in a non-endemic area. Data also indicated the existence of long-lived memory cells in the peripheral blood.

The duration of elevated antibody titres varied, serum vibriocidal antibody titres decreased gradually to baseline levels within 4-12 months, whereas antitoxin antibody titres remained elevated for longer periods (1 year up to 5 years). Despite the fact that antibody titres disappeared, sustained protection against cholera was demonstrated for 2 years in adults and for 6 months in children in the Bangladesh field trial. It should be remembered that these results were obtained in an endemic population where

natural boosters of cholera occur. The reason for the short-term protection in children is not known. In addition, protective efficacy differed by biotype, being inferior against El Tor cholera, which predominates on a global base.
The proposed recommendation to boost with one dose within 2 years after vaccination and to re-start a primary immunisation course if more than 2 years have elapsed is considered reasonable.

The recommendation for children of a booster dose after 6 months to maintain immunity against cholera is supported by the Bangladesh trial and is considered acceptable

Protective efficacy against cholera after booster vaccination has not been studied, which is mentioned in the SmPC.

Clinical efficacy

Main clinical studies

- **Efficacy in cholera**

The efficacy of the vaccine against cholera was assessed in 3 randomised placebo-controlled trials, Bangladesh field trial (Study 6), Peru military trial (Study 27) and Pampas field trial (Study 30).

In Study 6, the major cholera trial, 89,152 Bangladeshi individuals were included, of whom 63,498 received 3 complete doses given at 6 weeks intervals. Efficacy was followed up for 5 years. Both whole-cells (WC) and WC in combination with the native cholera toxin subunit B (WC/BS) were examined. Overall protective efficacy of the WC/BS vaccine in the primary population was 85% (95%CI 56,95) for the initial 6 months, regardless of age and severity of cholera. The BS component augmented efficacy during the initial 8 months, thereafter PE was similar for the WC/BS and WC vaccines During the first and second year protection was sustained in adults, but in the third year the efficacy was considered suboptimal (see table). Long-term protection differed notably by age and by biotype of the infecting agent, lasting for only 6 months in children and being superior for classical versus El Tor cholera. Sustained protection against classical cholera occurred also in children. An exploratory analysis suggested that 2 vaccine doses seemed as effective as 3 doses.

Table; Summary of vaccine efficacy in the Bangladesh field trial (study 6) against cholera after 3 doses (PP) in all subjects and children <6 years

Time after vaccination	**WC/BS vaccine** n=21 141		**WC vaccine** n=21 137		**Placebo** n=21 220
	Cholera cases	PE% (95%CI)	Cholera cases	PE% (95%CI)	Cholera cases
Adults >6 years					
6 months	4	**85** (56, 95) p=0.001	11	**58** (14, 79) p=0.017	26
Year 1	47	**64** (50, 74) p<0.001	58	**56** (39,67) p<0.001	131
Year 2	40	**52** (30, 67) p<0.001)	38	**55** (33, 69) p<0.001	84
Year 3	41	**19** (-22, 46) p=0.3	30	**41** (7, 62) p=0.022	51
Children <6 years	n=3 721		n=3 871		n=3800
6 months	0	**100**	6	**35** (-84, 77)	9
Year 1	27	**44** (10,65) p=0.016)	32	36 (0,59) p=0.049	49
Year 2	17	33 (-23, 64) n.s.	23		26
Year 3	23	<0	16	13 (-71,55) n.s.	18

It can be concluded that the WC/BS vaccine, in this well-conducted field trial in a cholera endemic area, conferred significant protection against cholera during the first 6 months and, in adults, moderate protection (~60%) for 2 years follow-up. Potential determining factors for the biotype- and age-related differences in the protective efficacy of the vaccine need to be further discussed.

In Study 27, enrolling 1,331 military recruits, the recombinant form of B subunit (WC/rBS) and a 2-dose schedule was used. Short-term PE of 85% (95%CI 38, 97) against El Tor cholera in this non-primed population was noted for the 5 months of follow-up.

While in these two key trials efficacy of WC/BS (Study 6) and WC/rBS (Study 27) could be demonstrated, the third big field trial (Study 30) failed to show efficacy during the 1st year. This trial involved 21,924 Peruvian volunteers given the WC/rBS in 2 doses two weeks apart followed by a booster dose after 10-12 months and with a total follow-up of 2 years. During the first year no protection was demonstrated, whereas for the second year, after the booster dose, 60.5% PE (95%CI 28,79) was achieved. The potential reasons for the unexpected lack of PE during the first year, included administrative errors and the use of active surveillance identifying also mild cases of cholera.

Supportive study(ies)
An early challenge study that used a preliminary vaccine not identical to the present composition provided proof of concept for the vaccine including whole-cells +/- BS. In this study, using WC+BS in a 3-dose regimen, vaccine efficacy of 64% was attained against a challenge with *V. cholerae* El Tor Inaba in previously unexposed US volunteers. The challenge was given 5 weeks after immunisation. Another challenge study, examining the present vaccine in a 2-dose schedule, was inconclusive due to insufficient activity of the challenge strain. A further field study conducted in Peru, including over 82,000 subjects, was unsuccessful due to a lack of cholera cases. In a 3-year follow-up of that trial, no protective efficacy of 2 vaccine doses was demonstrated against El Tor cholera.

Two feasibility studies suggested that the two-dose oral WC/rBS vaccine could be used for mass-vaccination in cholera epidemic areas. One of the studies included over 27,000 persons in a refugee camp in Uganda. The most important logistic problem identified was the bulkiness of vaccines and buffer solution required for the campaign, which complicated storage and shipment to vaccination sites

Data in other indications

Enterotoxigenic *Escherichia coli* (ETEC)

Rationale
Enterotoxigenic *E.coli* (ETEC) causes diarrhoea by first colonising the small intestine and thereafter producing one or more enterotoxins, the heat-labile (LT) and heat-stable (ST) enterotoxin. The LT enterotoxin is structurally, functionally and immunologically similar to the cholera toxin with an amino acid homology of approximately 80%. The two toxins cross-react immunologically and cross-protection has been demonstrated in animal models challenged with ETEC after immunisation with CT. Due to these findings, protective efficacy of Dukoral against LT-producing ETEC was investigated in human clinical trials.
Enterotoxigenic *E.coli* (ETEC) is the most common cause of bacterial diarrhoea in developing countries, responsible for up to 700 000 deaths yearly in children <5 years of age. The highest incidence of ETEC infection is in children less than 2 years of age, decreasing with age due to acquired immunity. The clinical spectrum includes mild self-limiting diarrhoea to severe cholera-like illness. ETEC is also the single most common pathogen causing traveller's diarrhoea (TD) and has been estimated to cause 5-18 million cases each year. The incidence of TD varies substantially with destination and season, but approximately 30-50% of travellers to Latin America, Africa and Asia experience diarrhoea and in 50% (20 to 75%) of these cases ETEC are isolated. The distribution of different enterotoxins among ETEC strains isolated in TD cases varies geographically, but approximately one-third of the strains are of the LT-only phenotype, 1/3 of the ST-only phenotype and 1/3 of the mixed LT+ST phenotype. Although a common health problem in travellers, TD is usually of mild to moderate severity and of short duration, average 3 days. Widespread use of antibiotics for prophylaxis and treatment of TD has led to the emergence of multiresistant ETEC.
There is a need for an effective ETEC vaccine with the main target group consisting of children <2 years of age in the developing world. A vaccine against ETEC would also be of benefit for travellers to high-risk regions.

- **Efficacy in LT-ETEC diarrhoea/Traveller's diarrhoea**

The trials specifically planned to demonstrate efficacy of the vaccine in the prevention of LT-ETEC diarrhoea (i.e. studies 9, 20, 22) did not unequivocally prove the protective efficacy. Study 9 was a Phase III study with European travellers (target population). The statistical significance of the study results was borderline (statistical significance depending on the method of analysis) and the treatment effects observed were not considered to be clinically convincing. Study 22 was a Phase III study enrolling US students travelling to Mexico. Due to problems in the study design (subjects were vaccinated upon arrival in Mexico) no protective efficacy of LT-ETEC could be shown. Study 20 was not conclusive as there was only one case of LT-ETEC in the study population. In Study 6, the BS-WC vaccine conferred borderline short-term protection against LT-producing ETEC. This protection (constituting a secondary endpoint only) was significant in the PP population but not in the ITT population. However, overall the incidence of ETEC diarrhoea was very low, raising concern about the robustness of the efficacy point estimate. As the study was performed in an endemic area the data could only provide mechanistic support for protection against LT-ETEC induced diarrhoea.

Clinical safety

Evaluation of the safety profile of the vaccine varied substantially between clinical trials with respect to the mode of surveillance, definition of symptoms and time of follow-up. In the large-scale studies, adverse events (AEs) were generally assessed by passive surveillance, which most likely have resulted in underreporting. The variation in incidence of AEs reported in different studies might relate to the methods of collecting data. No long-term follow-up of safety symptoms has been performed.

The safety of the new recombinant rCTB cholera vaccine was compared with the original CTB vaccine formulation in a randomised double blind study (study 11); *Vaccine 1992; 10: 130-132*)). The study population included Swedish volunteers aged 21-50 years, whereof 21 received two oral doses with a 2-week interval of the recombinant WC/RBS and 20 received the old WC/BS vaccine. In each of the vaccine groups a few of the volunteers reported mild GI symptoms. The safety data did not indicate any differences between vaccine groups.

Patient exposure

Over 240,000 subjects have been involved in clinical studies. Of these, over 127,000 received at least one dose of the WC/BS vaccine and approximately 30,000 received at least one dose of the WC component vaccine. The remainder received placebo or buffer only

Safety data are currently available for clinical studies involving 45,071 subjects (30,000 subjects received that CTB-containing vaccine and 15,000 received the rCTB vaccine) receiving at least one dose of the WC/BS vaccine including over 5,300 children aged 1-5 years. The native CTB containing cholera vaccine was used in the earliest studies (studies 1-14 and 17), including the Bangladesh field trial (study 6; n=21, 141 CTB cholera vaccinees), whereas the recombinant rCTB was used in the subsequent trials (studies 11, 15-48) including the Peru military trial (study 27; n=779 rCTB cholera vaccinees) and Pampas field study (study 30; n=9,012 rCTB cholera vaccinees). The safety data include populations from endemic (Bangladesh, Uganda), epidemic (Latin and Central America, Morocco), and non-endemic regions (Sweden, UK, USA,), men and women and different geographic areas. Vaccine tolerability was also studied in special populations, such as HIV infected individuals, IgA deficient subjects, peptic ulcer patients and colectomised subjects.

Adverse events and serious adverse events/deaths

The most commonly reported adverse events were gastrointestinal symptoms, including abdominal pain or discomfort, diarrhoea, loose stools, nausea and vomiting. These were observed with equal frequency between active and placebo groups. In most trials E.coli K12 was used as placebo but in some buffer only was used. Controlled trials showed that the GI symptoms could be attributed to the buffer. The symptoms were generally short lasting, self-limiting and did not require specific treatment. The safety profile was similar in children as in adults. No long-term follow-up of safety symptoms has been performed. There was long-term surveillance of vaccine recipients in the efficacy studies (Studies 6 and 30), but safety surveillance was only passive.

In the Bangladesh study there were 15 deaths, 5 in the BS-WC group, 8 in the WC group and 2 in the K12 group. No deaths were reported in studies 9 or 27, for which re-constructed study reports were available. No overall summary of number of deaths in the clinical programme has been provided. Safety data were collected by passive surveillance and therefore the 15 deaths represent grave underestimates. Published data from study 6 however are reassuring since a significant reduction in overall mortality was observed during the first year of vaccination. According to the company no serious adverse events related to the vaccine were reported during the trials. In the Bangladesh study, lists on hospital visits occurring within 13 days after each vaccination were provided.

The incidence of gastrointestinal symptoms varied across studies from 0% to 44%. A high incidence of 44% was observed in one study, involving 34 healthy adult US volunteers. In another trial conducted in military recruits in Peru, the incidence of stomach cramps was 28% after the first dose and 23% after the second dose. An incidence of 24% for GI symptoms was observed in a third study involving healthy adult military personnel. In other larger clinical studies, lower AE rates were recorded. The frequencies of specific symptoms reported such as loose stools and diarrhoea (2-14%), abdominal cramps (4.5-16%), nausea (0.4-5.0%), vomiting (0.6-1.8%), fever (0.8-1.2%) and headache (1.3-17%) varied, but occurred in similar frequencies in the placebo group. The rate and spectrum of AEs did not increase with subsequent immunisations.

During the PMS period (1992-Oct 2001) over 1 million vaccine doses have been distributed to approximately 500,000 travellers in the Scandinavian countries. A total of 72 AE reports have been reported in Sweden since 1992 and in Norway since 1998. Out of the 62 AEs with possible relationship to the vaccination, 29 were GI tract reactions, 7 were fever reactions, 6 were skin reactions (n=3 urticaria), 3 headache and 3 dizziness reactions. Comparing consecutive years during the reporting period revealed no evidence of an increased reporting of AEs.

Clearly there were deficiencies in the safety reporting of the clinical studies, which limits full assessment of the risk profile. However, since the vaccine have been in use for over ten years in Sweden and few adverse events and of serious grades have been reported, the vaccine can be considered safe.

Laboratory findings

N/A

Safety in special populations

No specific studies have been performed to assess safety of Dukoral in pregnant women, since the vaccine only contains inactivated and non-replicating components. The vaccine has been used in breast-feeding women in several studies without any adverse effects in relation to breast-feeding. Immunogenicity studies were performed in limited numbers of individuals with HIV-infection, IgA deficiency, peptic ulcer and those colectomised due to ulcerative colitis. No apparent safety issue was identified in these patient groups. A transient increase in viral load was observed after vaccination in the HIV-infected population.

5. Overall conclusions and benefit/risk assessment

The quality of this product is considered to be acceptable when used in accordance with the conditions defined in the SPC. Physicochemical and biological aspects relevant to the uniform clinical performance of the product have been investigated and are controlled in a satisfactory way.

No standard GLP studies on the pharmacology and toxicology of Dukoral have been conducted. From a scientific point of view, as discussed above, the paucity of preclinical studies is not considered a cause for concern. There are acceptable justifications for not carrying out comprehensive non-clinical investigations for this vaccine. Some of these justifications are also sufficiently supported by the relevant CPMP guideline on preclinical pharmacological and toxicological testing of vaccines (CPMP/SWP/465/05).

Overall, based on the particular characteristics of the vaccine and the clinical experience to date, additional preclinical studies are not warranted. The text in SPC sections 4.6 and 5.3 is appropriate and accurately reflects the lack of non-clinical data. Efficacy and the overall safety of the vaccine should be judged on clinical data.

In addition recital 10, of Annex I of Directive 2001/83/EC, as amended states: " However, there are reasons of public policy for not conducting repetitive tests on humans or animals without over-riding cause". Reference is also made to Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes, and Council Decision 1999/575/EC of 23 March 1998 concerning the conclusion by the Community of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Two trials support a 2-dose schedule (Study 6 and 27) whereas the Pampas trial (Study 30) indicates that 3 doses would be needed. However, the administrative problems associated with Study 30 make these results less reliable. Besides the PE of the vaccine reached in studies 6 and 27, serological data supported a 2-dose primary regimen. Moreover, the memory responses induced by 2 doses were of the same magnitude as after 3 doses. The Company has not investigated a 3-dose primary schedule followed by a booster after 1 year and no data on this potentially improved regimen exist. Thus, available data support a 2-dose primary regimen in adults. Antibody titers against both the CTB and the whole-cell component increased substantially by day 7 as demonstrated in a compilation of study data.

Thus, the claimed primary 2-dose vaccination schedule and the timing of the booster dose for adults were accepted**.** For younger children <6 years short term protective efficacy was demonstrated; after 3 doses of BS-WC the protection waned rapidly within 1 year. The recommendation for children to give a booster dose after 6 months was considered acceptable.

The vaccine could be used both for individualised as well as for population immunisation, but for Europe the intended target group is travellers to endemic areas. Immunogenicity studies were performed in naive EU subjects, but no pivotal efficacy trial was conducted in this population. The risk of cholera for regular tourists is minor, whereas in certain groups, such as health care workers in epidemics, the vaccine could be of great importance.

In an oral explanation in front of the Committee the applicant further discussed the findings in the trials performed in travellers' diarrhoea. The existence of only one pivotal trial in the target population for travellers' diarrhoea with results of borderline statistical significance was not considered sufficient for recommendation of the granting of a Marketing Authorisation. The Committee recommends a statement in Section 5.1 of the SPC on the structural, functional and immunological similarities between heat labile toxin of enterotoxigenic E. coli and the B-subunit of the cholera toxin.
The risk profile of the vaccine is favourable.
The overall benefit risk relationship was considered favourable for Dukoral in the indication for active immunisation against disease caused by *V. cholerae* serogroup O1 of adults and children from 2 years of age.

Table of all clinical trials with Dukoral

Abbreviated Name **rCTB/CTB**	**Location, year***	**Clinical Phase**	**Volunteers (n)**	**Age and Sex**	**Objective(s)**	**Reference/Report**
1. Gbg Dose I CTB	Bangladesh, 1980	I	34	Adult men and women	Safety and immunogenicity of 2 doses of CTB compared with cholera convalescents	Svennerholm, A. M et al 1984. J Infect Dis 149:884-93 Jertborn, M., et al. 1986. J Clin Microbiol 24:203-9.
2. Five-year immunologic memory CTB	Sweden, 1982-87	I	27 total 15 primary, 12 booster	Healthy adults	Immunogenicity of a booster dose given 2.5-5 years after primary vaccination	Jertborn, M., A. M. Svennerholm, and J. Holmgren. 1988. J Infect Dis 157:374-7
3. Gbg Dose II CTB	Sweden	I	27	Adult men and women	Safety and immunogenicity of 2 doses of CTB. Same as #1.	Jertborn, M., et al. 1984. Int Arch Allergy Appl Immunol 75:38-43.
4. Neutralization of gastric acid CTB	Bangladesh 1984	II	196 total (143 vaccine, 53 buffer only)	Children 2-14 years Women >14 years	To assess if the oral BS/WC vaccine requires protection from gastric acid	Clemens, J. D., et al 1986. J Infect Dis 154:175-8.
5. Pre-Bangladesh CTB	Bangladesh, 1984	II	1257 total: 898 took 3 doses	Children 2-15 Women >15	Safety and immunogenicity of whole cell ± CTB	Clemens, J. D et al. 1987. J Infect Dis 155:79-85.
6. Bangladesh CTB	Bangladesh, 1985	III	89152 total 63 498 took 3 doses	Children 2-15 Women >15	Efficacy (anti-cholera and anti-ETEC)	Study report: CSR-010
7. 1-year ASC CTB	Sweden 1987	I	5	Adults (25-55 years)	Immunogenicity: ASC 1 year after vaccination	Lycke, N., et al. 1987. Scand J Immunol 26:207-1
8. IgG and IgA subclass CTB	Sweden and Bangladesh	I	15-21 overlap #3	Adult men and women	Subclass distribution after vaccination and disease	Jertborn, M., et al. 1988. Int Arch Allergy Appl Immunol 85:358-63.
9. Peltola CTB	Morocco, 1989	III	615	Adult Finnish tourists	Efficacy (anti-ETEC)	Study report: CSR-011 Peltola H et al. Lancet Nov 23, 1991; 338: 1285-1289.
10. ASC and gamma-interferon CTB	Sweden 1989-90	II	10	Male and female aged 26-49 years	Local antibody secreting cells and local cytokine production	Quiding, M., et al 1991. J Clin Invest 88:143-8.
11. Gbg CTB CTB and rCTB	Sweden, 1990-1991	I	41	Adults 21-50	Safety and immunogenicity of BS-WC *versus* rCTB-WC	Jertborn M. et al. Vaccine 1992; 10: 130-132.
12. Gbg Dose Interval CTB	Sweden, 1990-1991	I	180	Adults	Effects of number of doses, dose interval and coadminstered buffer	Jertborn M. et al. Vaccine 1993; 11: 1007-1012.

*In those cases when the study year is not known, the year of the publication is used.

Abbreviated Name	Location, year	Clinical Phase	Volunteers (n)	Age and Sex	Objective(s)	Reference/ Report
13. Cell responses booster CTB	UK 1991 (publ)	I	13	Male adults	Immunogenicity (ASC)	Lewis, D. J et al. 1991. Eur J Immunol 21:2087-94.
14. HIV Sweden CTB	Sweden, 1990-1992	II	8 HIV 10 healthy	Men and women age 36-65	Safety and immunogenicity in HIV-infected volunteers	Eriksson, K. et al. 1993. Aids 7:1087-91.
15. Fort Bragg I rCTB	USA 1991	II	74	Military personnel 18-44 years	Safety and immunogenicity,	Sanchez, J. L., et al. 1993. . J Infect Dis 167:1446-9.
16. Fort Bragg II rCTB	USA 1991	II	186	Military personnel 18-44 years	Safety and immunogenicity, 2 different buffer doses	Sanchez, J. L., et al. 1993. . J Infect Dis 167:1446-9.
17. Gbg Memory rCTB	Sweden, 1991-1992	I	66	Adults 18-48	Immunological memory 10 months after vaccination	Jertborn et al. Vaccine 1994; 12: 1078-1082.
18. Ancon rCTB	Peru 1992	II	346	Military recruits Men 17-23 years	Safety and immunogenicity, Comment: Cholera epidemic immediately after trial began.	Sanchez, J. L. et al.1995. Trans R Soc Trop Med Hyg 89:542-5. Short report available
19. Chilean children rCTB	Chile 1992	II	485 total	Children 2-15 years	Safety, immunogenicity, 2 doses, varying dose interval 1-6 weeks. Booster dose after 6 months. Not blinded.	Not published, RA Kuschner IND annual report available
20. Mediterranean shipboard rCTB	US 1992	III	1225 3-dose recipients	Adults >18 years	Efficacy against ETEC diarrhea. 2 doses and booster after 3 months. No cases, they went to Yugoslavia instead of Egypt.	Not published. S. J. Savarino. IND summary available.
21. Barranquilla rCTB	Colombia, 1992	II	1165 2-dose recipients	Children and adults 1-64 years	Safety and immunogenicity of the WC/rBS vaccine in Colombian volunteers aged 1-64 years.	Alberto Concha et al. 1995. Bulletin of PAHO. 29(4): 312-321.
22. US students in Mexico rCTB	Mexico 1992	III	502	Adults (US college students)	Safety, immunogenicity and protective effiacacy against ETEC diarrhea when vaccinating after arrival.	Ernesto G. Scerpella, et al. 1995. J. Travel. Med. 2:22-27.
23. HIV UK and Kenya rCTB	UK and Kenya 1992-93	II	20 UK healthy 12 UK HIV 20 Kenyan HIV	Men and women 26-52 years	Safety and immunogenicity in HIV-infected volunteers	Lewis, D. J. et al 1994. Aids 8:779-85.
24. Challenge rCTB	USA 1993	III	18	Men and women 18-40 years	Efficacy after challenge with *V. cholerae*.	Not published Taylor IND summary available.

Abbreviated Name	Location, year	Clinical Phase	Volunteers (n)	Age and Sex	Objective(s)	Reference/ Report
25. IgA+G deficient rCTB	Sweden 1993 (publ)	II	23 IgAd 11 normal	Adult Men and women	Immunogenicity	Nilssen, D. E., et al. 1993. Scand J Immunol 38:201-8. Nilssen, D. E, et al. 1993. Immunodeficiency 4:55-7
26. IgA deficient rCTB	Sweden 1994 (publ)	II	30 IgAd, 21 healthy volunteers	Adult Men and women	Safety and immunogenicity in IgA deficient volunteers	Friman, V.et al. 1994. Clin Exp Immunol 95:222-6.
27. Peru Military RCTB	Peru, 1994	III	1331	Adults 17-65	Efficacy (anti-cholera)	Study report CSR-003 Sanchez, J.L. et al. Lancet 1994; 344: 1273-1276.
28. T lymphocytes RCTB	UK 1993-1995 (publ)	I	Approx 10	Adults	T-cell mediated immunity	Castello-Branco, L. R., et al. 1995. Vaccine 13:817-20. Castello-Branco, L. R. et al. 1994. Vaccine 12:65-72. Lewis, D. J. et al. 1993. Vaccine 11:119-21.
29. El Carmen RCTB	Peru, 1993-1994	II	541	Children and Adults 2-65	Safety and Immunogenicity after 2 primary and a booster dose after 1 year.	Begue, R.E. et al. Vaccine 1995; 13: 691-694. Begue, R.E. et al. Infect. Immun 1995; 63: 3726-3728.
30. Pampas de San Juan rCTB	Peru, 1993-1995	III	35 554 total 15 026 3-dose recipients	Children and Adults 2-65	Efficacy (anti-cholera)	Taylor, DN et al. J Inf Dis 2000;181:1667-1673.
31. Egyptians and Americans rCTB	Egypt, 1993-95	II	120 Egyptians 21 US volunteers	60 Egyptian 2-5 years and 60 adults 21 US adults	Safety and immunogenicity in different populations	Full CSR by D. Tribble. (IND, Not by SBL) Not published
32. Arequipa rCTB	Peru 1994-98	III	92749 total 82762 3-dose recipients	Children and Adults 2-65	Efficacy against cholera in a Peruvian population	C. Lanata, Not published,
33. Helicobacter pylori- infected rCTB	Sweden 1994-96	II	19	Men and women aged 23-50	Safety and immunogenicity in *H. pylori*-infected subjects	Mattsson, A1998. J Clin Invest 102:51-6.
34. Ulcerative colitis rCTB	Sweden 1994-97	II	35	Men and women aged 22-73	Safety and immunogenicity in ulcerative colitis patients	Kilhamn, J., 1998. Infect Immun 66:3995-9.
35. ASC characterization rCTB	Sweden 1995 (publ)	I	14	Healthy adults	Characterization of B cell surface markers after oral or parenteral (TT) vaccination	Lakew, M. et al. 1995. Adv Exp Med Biol 371B:1451-3.

Abbreviated Name	Location, year	Clinical Phase	Volunteers (n)	Age and Sex	Objective(s)	Reference/Report
36. ASC in tonsils rCTB	Sweden 1995 (publ)	I	7 oral	Healthy adults (16-37 years)	Induction of tonsillar B-cell responses after oral or other route	Quiding-Jarbrink, M., et al. 1995. Infect Immun 63:853-7.
37. ASC homing receptors rCTB	Sweden 1995 (publ)	I	23	Healthy men and women 22-51 years	Characterization of B cell homing receptors after oral or parenteral (TT) vaccination	Quiding-Jarbrink, M. et al. 1995. Eur J Immunol 25:322-7.
38. Susana Higushi rCTB	Peru, 1995	II	164	Children and Adults 2-65	Safety and Immunogenicity	Taylor N D et al. Am J Trop Med Hyg, 61(6), 1999, 869-873.
39. HIV Brazil rCTB	Brazil, 1995	II	12	Men aged 30-60	Safety and immunogenicity in HIV-infected volunteers	Ortigao-de-Sampaio, M. B., 1998. Aids 12:F145-50.
40. Acetylcysteine rCTB	Sweden 1995-96	II	40	Men and women aged 23-53	Kinetics of local and systemic immune responses after vaccination alone or together with acetylcysteine	Kilhamn, J., et al 1998. Clin Diagn Lab Immunol 5:247-50.
41. US and Mexicans rCTB	Mexico 1996 (publ)	I	10 US 18 Mexican	Men and women >18 years	Immunogenicity and kinetics of immune response	Scerpella, E. G. et al. 1996. J Travel Med 3:143-147.
42. Vaginal and oral vaccination rCTB	Sweden 1996 (publ)	I	7 oral	Adult women	Comparison of immune responses after vaginal and oral vaccination	Wassen, L. et al. 1996. Scand J Immunol 44:408-14
43. Oral, nasal and systemic vaccination rCTB	Sweden 1997 (publ)	I	14 oral	Healthy adults, 18-51 years	Comparison of cell markers after oral, nasal and systemic vaccination	Quiding-Jabrink, M. et al. 1997. J Clin Invest 99:1281-6.
44. Uganda RCTB	Uganda 1997	Feasibility	27 607	>1 year Men and women	Feasibility of mass vaccination in a refugee camp	Legros, D., et al. 1999. Bull World Health Organ 77:837-42. Dorlencourt, F.et al. 1999. Bull World Health Organ 77:949-50.
45. OCV-028 RCTB	Nicaragua, 1997	II	125	Children aged 1-12 years	Safety and immunogenicity in Nicaraguan children	Study report, draft Not published
46. Oral, rectal, vaginal vaccination RCTB	USA 1997 (publ)	I	14 (total, oral, rectal or vaginal)	Healthy adults, women	Immunogenicity, comparison oral, rectal, vaginal vaccination	Kozlowski, P. A et al. 1997. Infect Immun 65:1387-94.
47. Nasal kinetics rCTB	Sweden 1997	I	9 oral	Healthy adults women	Immunogenicity, comparison oral vs. nasal vaccination	Rudin, A., et al. 1998. Infect Immun 66:3390-6.
48. Urinary tract rCTB	Sweden 1997	I	6 oral	Healthy adults men	Immunogenicity, comparison oral vs. nasal vaccination	Rudin, A., et al. 1999. Infect Immun 67:2884-90.