

Bovine hyperimmune whey protein concentrate with specific biological activity as a replacement ingredient

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The progress made in understanding the underlying mechanisms of immunity, appearance of antibiotic resistant bacteria and an increasing number of gastro-intestinal (GI) tract originated infections has provoked renewed interest in the development of immune milk preparations for prevention, or treatment of microbial infections in humans. MucoVax is a biotechnology company (est. 1998) that specializes in the elicitation of immunoglobulins (Ig), with an emphasis on S-IgA production, in milk of dairy animals for nutritional therapy and risk reduction or prevention of gastro-intestinal infections in humans. The Ig are directed against pathogens of the human GI tract, applied orally and have a beneficial effect either direct or in combination with a conventional treatment.

MucoVax's platform technology is based on the mucosal immune system of the cow. The Ig composition of milk, produced by the mammary gland, represents the different pathogens that challenged the animal during its life. In this way protecting its offspring against the environmental pathogens. By challenging the cow's natural defence system with vaccines containing pathogen-derived antigens, specific Ig production can be elevated in the milk. Normal breeding and selection techniques are applied and the health condition of the animal is a prerequisite for the expression of antibodies in the milk. By using milk instead of bovine colostrum MucoVax elegantly circumvents problems of logistics and up scaling of the technological processes. From a commercial point of view the profitable exploitation of polyclonal antibody preparations from milk of immunised cows has not the limitations of colostrum, which can only be collected just after calving for the first 3 days. Two tier products are currently in the pipeline. Mucovax's first tier product is a natural bovine hyperimmune whey product containing specific Ig against *Clostridium difficile* and its toxins as a replacement ingredient for the application as nutritional supplements, in functional foods and in clinical nutrition.

The second tier product is a nutritional therapy based on the same technology to prevent enterotoxigenic *Escherichia coli* (ETEC)-associated travellers' diarrhoea. Travellers' diarrhoea affects 30-70% of persons travelling to (sub)tropics.

The experimental farm of MucoVax is a closed Dutch farm; cows are free of infectious diseases. Milk production complies with Dutch quality control standards. Production of world-wide applications will be organised by breeding and selecting special herds in closed farm units of at least 500 lactating animals.

MucoVax has several collaborations with research institutes and universities. But MucoVax's partnering goal is to have a strategic alliance with one or two suitable international innovative food industries or ingredient industries for commercialisation of its products.

Working with MucoVax offers:

- Mucosal immunology competence centre
- Expertise in hyperimmunisation strategies of cows
- An experimental farm
- Option for production
- Scientific advisory board of leading experts
- Efficacy/food safety demonstrated by pre- and clinical research
- Nutritional therapies and preventive nutritional support for gastro-intestinal infections in humans

2. Background of *Clostridium difficile* - associated diarrhoea

C. difficile associated diarrhoea (CDAD) is a common, iatrogenic, nosocomial disease, associated with substantial morbidity and mortality, each year causing an estimated 3,000 deaths in the U.S. Recurrence of CDAD is a substantial clinical difficulty (Johnson and Gerding, 1998). *C. difficile* is an obligate anaerobic, spore-forming, Gram-positive micro-organism. Some strains of this naturally in the environment occurring organism form potent toxins, an enterotoxin (toxin A) and a cytotoxin (toxin B) (Sears and Kaper, 1996). Toxin producing strains are the causative agent of CDAD. CDAD occurs predominantly in hospitalised patients on antibiotic treatment and senior citizens in geriatric/rehabilitation wards (Fig. 1) (Wilcox and Minton, 2001; Kyne *et al.* 2001). It is estimated that there are approximately 1.5 million cases of CDAD in the US each year. A conservative estimate of the cost of this disease in the US is in excess of \$1.1 billion per annum (Kyne *et al.*, in press).

Infections have become endemic in many hospitals in the UK and yet few data on the associated costs of such cases are available.

Wilcox *et al.* (1996) published data concerning the financial burden of hospital-acquired *C. difficile* infections in the UK. The total identifiable increase in costs due to the fact that patients are hospitalised longer (approximately 18 days) exceeds the 4000 Pounds. Most patients with CDAD respond well to medical therapy that includes discontinuation of the inciting broad-spectrum antibiotic and treatment with metronidazole or vancomycin. However, despite successful treatment of initial episodes, recurrence of diarrhoea after withdrawal of specific antibiotic therapy is a substantial clinical difficulty. Recurrence rates of 5-65% have been reported, dependent on definition of recurrence and population studied. Active or passive immunisation against *C. difficile* could be effective, not only in preventing initial episodes, but also in reducing risk of recurrence. However, production of a vaccine for active immunisation that is immunogenic in the elderly, especially those in whom natural challenge with *C. difficile* does not elicit a protective antibody response, is a daunting response. Use of passive immunization is likely to be revisited, particularly for those patients with severe or multiple recurrences of *C. difficile* infection.

3. Results

3.1 Immune response in the milk of lactating cows

MucoVax developed an immunisation

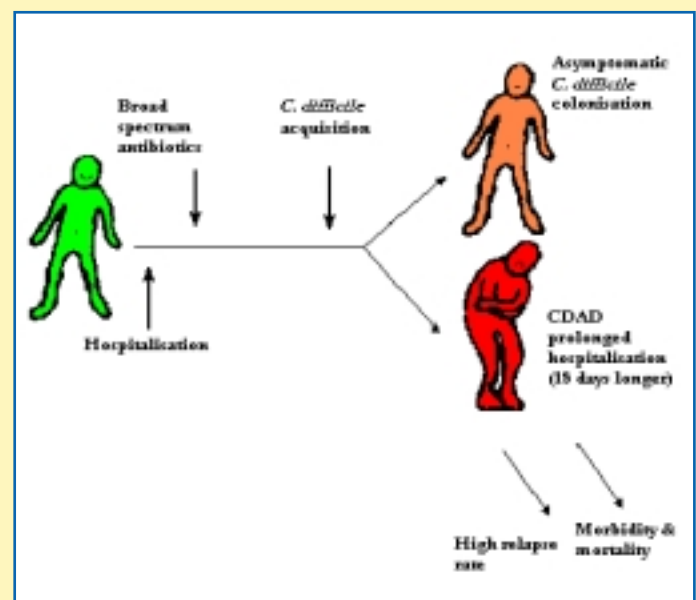


Fig. 1: A hospitalised person is intermittently exposed to *C. difficile* throughout his/her hospitalisation but does not become highly susceptible to *C. difficile* infection until after receiving a broad spectrum antibiotic therapy. After a very brief incubation period following infection, the clinical outcome is determined.

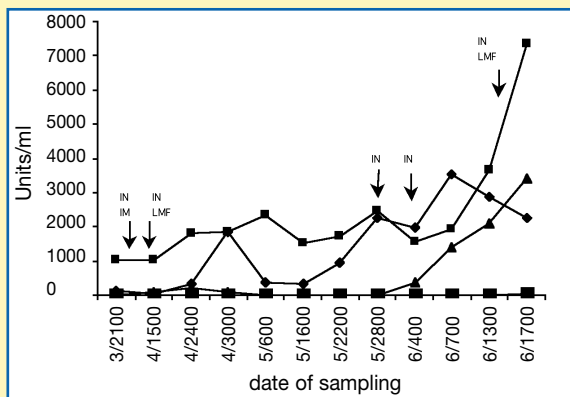


Fig 2: A graphic illustration of anti-*Clostridium difficile* IgA responses in the milk of hyperimmunised cows (versus 1,000 units/ml of the standard colostrum of hyperimmunised cows). Different routes of antigen administration used i.e. IN=intra-nasal; LMF=lymph; IM=intramuscular

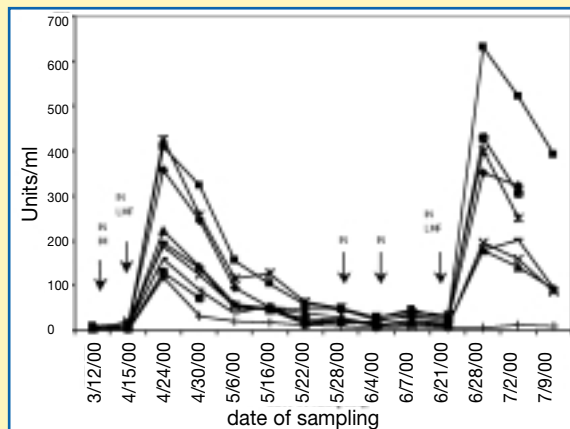


Fig 3: A graphic illustration of anti-*Clostridium difficile* IgG responses in the milk of hyperimmunised cows (versus 1,000 units/ml of the standard colostrum of hyperimmunised cows). Different routes of antigen administration used i.e. IN=intra-nasal; LMF=lymph; IM=intramuscular

strategy sensitising the cow's mucosal and systemic immune system for *C. difficile*. Immunogen-specific immunoglobulin IgA levels in the lactogenic secretions exceeded in more than 50% of the animals the normal colostrum levels. Immunogen-specific IgG reached at least the same level of specific Ig found in colostrum of hyperimmunised cows (Figs. 2 and 3). The immunisation protocols have a minimal invasive character and the life-stock is under a continuous observation of a veterinarian.

3.2 Efficacy of bovine hyperimmune whey protein concentrate in a HeLa cell line assay

In this assay (Fig. 4) *C. difficile* toxins cause disruption of the intracellular filaments of target cells (HeLa), resulting in rounding of cells and cell death (Kelly et al., 1996). This process can easily be monitored with a light microscope. Cultured HeLa cells were exposed to varying doses of pure toxins (10 pM to 100 nM) for 24 h. Percentage of cell rounding was measured at 24 h in the presence or absence of the whey protein concentrate. The results are expressed as the reduction of cytotoxicity required to neutralise 8 toxic doses of toxin A or B, defined as the least amount of toxin required to cause 50% cell rounding of HeLa cells at 24 h (Table 1). The protective properties against *Clostridium difficile* toxin A and toxin B of the anti-*C. difficile* hyperimmune whey protein concentrate have been clearly demonstrated. The toxicity of toxin A

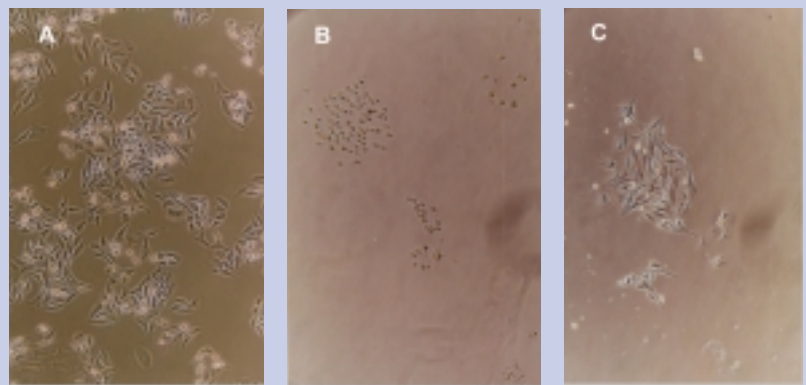


Fig. 4: Toxin neutralizing HeLa cell line assay with bovine hyperimmune whey protein concentrate

- A. Untreated HeLa cells
- B. HeLa cells after being subjected to *C. difficile* toxins
- C. Toxin neutralisation by bovine hyperimmune whey protein concentrate. No cell death occurred.

is reduced by 40 times and toxin B by 332 times (Table1).

3.3 Efficacy of hyperimmune bovine whey protein concentrate in experimental animals

To demonstrate efficacy of the hyperimmune bovine whey protein concentrate in animals, the clindamycin-induced-colitis hamster model was used. The hamster model offers clear end points, is reproducible and the disease manifestations such as diarrhoea and colitis are similar to those found in humans. Hamsters were primed with clindamycin (a broad-spectrum antibiotic) to reduce

diarrhoea and survival during this period. After four days of treatment the animals that survived, were observed for an additional two weeks for monitoring occurrence of relapse. In group 4: 90% of the animals survived and in group 3 all animals died. The survival rate in group 4 was significantly higher than in group 3 ($p < 0.05$) (Warny, 2001). Another interesting finding is that the animals in group 4, survived for more than two weeks after discontinuation of the hyperimmune whey administration after day 4 and showed no relapse of infection before they were sacrificed. Bacterial analysis of stool specimens is performed now to investigate whether the survivors are still carriers of the particular *C. difficile* strain used in the experiments. It was concluded that hamsters treated with

Table 1: Reduction of cytotoxicity in HeLa cell line by hyperimmune whey protein concentrate

	Control whey protein concentrate	Hyperimmune whey protein concentrate	Reduction of cytotoxicity
Toxin A	15 ng/ml	600 ng/ml	40 x
Toxin B	30 pg/ml	10 ng/ml	332 x
Control = 100 % cell death			

the colonic flora and infected with *C. difficile* orally to induce acute colitis. This model gives a mortality rate of 95-100% within 72 h. Bovine hyperimmune whey protein concentrate was tested on its ability to reduce mortality rates. Four groups of ten hamsters each were used. Group 1: negative control to monitor the hamster colony for any external cause of diarrhoea or death; group 2, 3 and 4 were infected with *C. difficile* and received different treatments. Group 2 did not receive the bovine hyperimmune whey protein concentrate; group 3 received control bovine whey protein concentrate of non-immunized cows, whereas group 4 received the bovine hyperimmune whey protein concentrate. The whey was orally administered with a feeding tube. Three hours before inoculating the bacteria, the hamsters were given 1 ml of whey. Three hours after infection, animals were given whey for three consecutive days (1 ml every 8 h) and were monitored for

bovine anti-*C. difficile* hyperimmune whey protein concentrate were protected against *C. difficile*-induced lethal intestinal inflammation. These findings suggest that orally administered anti-*C. difficile* hyperimmune whey protein concentrate may be beneficial for persons suffering from a *C. difficile* infection. In addition, passive immunisation may be beneficial for treating immuno-compromised patients with chronically relapses of this disease. Table 2 shows the reduction of mortality of various other preparations in the *C. difficile*-associated colitis hamster model. MucoVax hyperimmune whey preparation shows the highest efficacy compared to the other preparations.

3.4 Resistance of anti-Clostridium difficile nutraceutical in human gastrointestinal (GI) tract

In order to be therapeutically active against *C. difficile* in the GI tract, hyper-

Table 2: Percentage survival in *C.difficile* colitis in hamster study: a comparison

Preparation	1st Dose before <i>C.difficile</i> challenge	Oral dose (Od)	Total treatment	Reduction mortality
MucoVax Hyperimmune whey (total Ig)	-3h	1-mg/Od 2xOd at day 1; 3x Od at day 2,3,4	11 mg	90% (n=10) no relapse ³
IgG from Colostrum ¹	-48h	300-mg/Od 3xOd daily during 13 days	11700 mg	70% (n=9) relapse
IgY (Chicken egg yolk) ²	-24h	40-mg/Od 3xOd daily during 7 days	840 mg	100% (n=7) no relapse

¹ Lyerly et al. (1991)² Kink et al. (1998)³ No relapse of infection for more than 2 weeks after discontinuation of the hyperimmune whey administration after day 4

immune bovine whey protein concentrate must resist digestion and reach the colon intact. This part of the study will be performed in the TNO dynamic, multi-compartmental system of the stomach and small intestine (Fig. 5 TIM-1)(Minekus and Havenaar, 1998). This model simulates very closely the successive dynamic conditions in the gastric-small-intestinal tract, such as body temperature, pH, concentrations of electrolytes, enzymatic activity in the stomach and small intestine, bile salts concentrations in the different parts of the gut, kinetics of chyme passage through the stomach and small intestine, and the absorption of low molecular weight molecules and water. The bovine hyperimmune whey protein concentrate is now tested for stability in the TIM-1 model. Under average physiological conditions as described for human adults during fasting state, after intake of food, and/or water with bicarbonate the activity of the immunoglobulins in the whey preparation will be

measured by ELISA and toxin neutralisation HeLa cell line assay.

3.5 Clinical case studies: efficacy / food safety

For demonstrating efficacy and food safety, a clinical case study will start in January 2002 in the Netherlands. The first aim of the Dutch study in hospitalised patients treated for *C.difficile* diarrhoea and colitis, is to establish whether hyperimmune whey containing specific immunoglobulins raised against *C. difficile* and its toxins, can be applied as oral adjunct after completion of standard antibiotic treatment with metronidazole or vancomycin, to prevent a relapse of *C. difficile* colitis. The second aim of the study is to examine whether the hyperimmune whey can eliminate carriage or prevent patient-to-patient transmission of *C. difficile*. In a later phase, similar clinical case reports will be generated in US, UK and France by local clinical investigators.

3.6 Product development

A cost-effective industrially scalable manufacturing process is currently being developed for the isolation of S-IgA/IgG from hyperimmune milk at a scale of 1000 litres per day. This production process can be applied at

production farms with even higher throughput volumes. A purity level of 85-90% can be achieved with modern purification procedures and the concentration of residual whey proteins is kept at a very low level with these techniques. It should be noted that the nature of the impurities is more important than the degree of purity itself. There is no need to purify the end product to a level that is needed for parenteral administered protein preparations because the S-IgA/IgG from bovine milk will be registered in the US as a Generally Recognized as Safe (GRAS) product. Notwithstanding the lower standards for nutraceutical protein preparations MucoVax's production process yields a product that can meet the high quality demands asked for in pharmaceutical preparations. Fig. 6 shows an SDS-PAGE experiment, early in the purification development process, with protein profiles of a commercial available bovine IgG fraction compared to a bovine anti-*C.difficile* hyperimmune whey protein concentrate and the purified total pool of immunoglobulins (IgM, IgG and S-IgA) from this whey. The purified total immunoglobulin pool isolated from the hyperimmune milk has a purity of 85-90% based on SDS-PAGE.

4. Conclusion and outlook

Consumption of anti-*C.difficile* hyperimmune whey protein concentrate could prevent occurrence and/or reduce the risk of recurrence of CDAD. Efficacy testing is currently in progress in clinical trials with patients. Market acceptance of MucoVax first tier product, a bovine hyper immune whey protein concentrate against *C. difficile*, will be positioned as a nutraceutical or food ingredient with the GRAS status. Promising applications of MucoVax's first tier product is twofold.

- A whey product with high biological value for application in new or line extensions of existing clinical nutrition products or nutritional supplements and positioned for an effective nutritional therapy of people with CDAD.
- A whey product with high biological value in a lower concentration in functional foods for risk reduction. Possibly in combination with micro-nutrients, antioxidants, insoluble fibres, pre- and probiotics for preventive nutritional support in order to raise protection of elderly in rehabilitation /geriatric wards against outbreaks of CDAD.



Fig. 5 Picture of the TNO dynamic model of the stomach and small intestine (TIM-1)

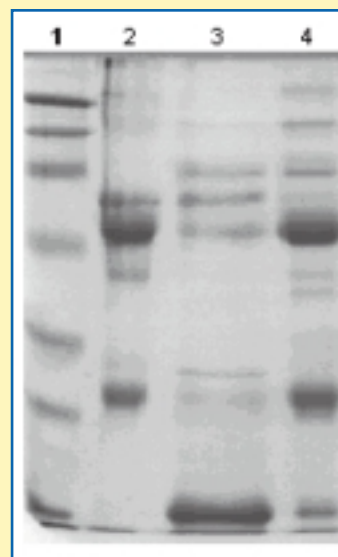


Fig 6: SDS-PAGE 12.5%.

Lane 1: Mr markers, Myosin 201 kDa, β -galactosidase 130kDa, Bovine serum albumin 94 kDa, Ovalbumin 48.6 kDa, Carbonic anhydrase 36.4 kDa, Soybean trypsin inhibitor 29.8 kDa, 36.4 kDa, Soybean trypsin inhibitor 29.8 kDa, Lysozyme 20.6 kDa, Aprotinin 6.6 kDa;
Lane 2: commercial affinity purified bovine IgG (10 microgram);
Lane 3: whey from milk (10 microgram);
Lane 4: MucoVax total pool Ig (10 microgram).

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Internet answers to global food safety risks

The development of the Internet has, for the first time, given the food sector the ability to develop common standards of hygiene and safety right across the globe, industry representatives heard in September.

Delegates at the CIES International Food Safety Conference in Geneva were told how the worldwide web has now made possible the global exchange of information in real time which is vital for the effective management of food safety risks.

The power of the web in enabling food producers, processors, packers and retailers to achieve greater control over food safety was outlined to the high profile gathering by Dr Esteban Delgado, International Technical Director of risk management specialists, The National Britannia Group.

He was addressing the plenary session of the special two-day conference called to discuss strategies for achieving food safety standards which can be applied across all countries.

The event has been organised by the Paris-based CIES The Food Business Forum, as part of its Global Food Safety Initiative launched last year to develop standards to enhance food safety in order to ensure consumer protection and maintain consumer confidence.

CIES represents around 250 food retailers and a similar number of suppliers, drawn from a total of 48 countries. These businesses have combined turnover of \$2,800 billion with the retailers accounting for 600,000 stores, employing a total of four million people.

Dr Delgado said in his presentation: "Globalisation of the food trade has given the benefits of choice to consumers but it has also underlined the need for food safety standards which apply everywhere in the world."

He pointed out that the World Health Organisation Codex Alimentarius Commission favoured a "risk based" approach to achieving food safety objectives, which meant controlling safety at every critical point in the food chain.

"This is where the use of web based tools for food safety management can deliver huge advantages and give the industry an unprecedented degree of control," he added.

Said Dr Delgado: "The implementation of risk-based systems involves phases where monitoring and verification are critical for ensuring that control procedures are in place and risks are minimised.

"To date the use of software packages and databases have proved very useful for managing the information coming from such monitoring activity but they have severe limitations in that the exchange of information between all stakeholders is restricted by systems incompatibility between different bodies, companies and institutions."

Using the Internet overcomes these barriers, allowing key information to be shared across the world in real time using common

web browsers, Dr Delgado explained.

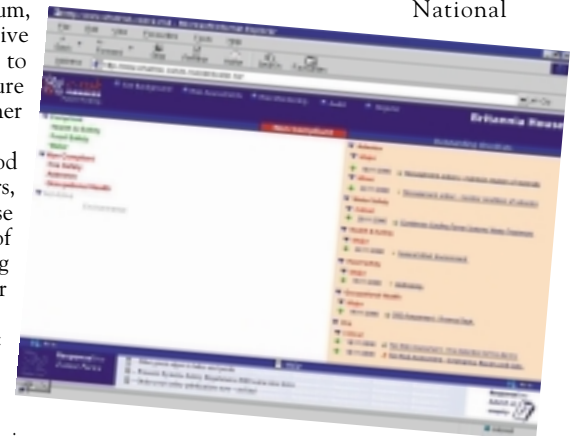
"The development of web based tools for food safety management allows the integration of internal monitoring procedures, external audit reports and databases and interactive management systems," he added.

By using such integrated systems, multi-site businesses, such as retail chains, can monitor the food safety compliance of their own operations or the compliance of suppliers or third parties anywhere in the world.

"Compliance status, audit reports, principal deficiencies and database management can all be viewed and operated from a central location in real time. This has been shown to improve food safety through greater time efficiency and faster response to identified problems, wherever they arise," he added.

In addition to better risk management, the Internet can contribute further to food safety improvements by delivering standardised on-line training to food handling staff at sites across the globe.

Dr Delgado and colleagues from the National



Britannia research and development team have been working with partner organisations in Europe and North America over the past two years to develop a range of Internet based risk management systems.

This has led to the creation of the eRisk MANAGER system with a special food safety module which contains interactive sections tailored for the needs of food retail stores, food retailer supplier management and food processors.

