Aanvraagformulier

Beoordeling van veterinaire toepassingen gentherapeutica

-

Virale vectoren

INHOUDSOPGAVE

1. Algemene gegevens aanvraag

Algemene gegevens

1.1. Titel van de aanvraag:

Vaccination of chickens with a herpesvirus of turkey vaccine with inserted the F-gene of Newcastle Disease Virus and the VP2 gene of Infectious Bursal Disease Virus.

1.2. Het doel van de werkzaamheden die worden aangevraagd:

Purpose is to investigate under field conditions the safety and efficacy of the HVT-ND-IBD vaccine (herpesvirus of turkey (HVT) with inserted the F-gene of Newcastle Disease Virus (NDV) and the VP2 gene of Infectious Bursal Disease Virus (IBDV).

1.3. Geef een korte inhoudelijke beschrijving van de aanvraag, het nagestreefde belang van het onderzoek en de beoogde toepassing van de resultaten.

GMO:

The HVT recombinant vaccine with inserted the F-gene of Newcastle disease virus (NDV) and the VP2 gene of infectious bursal disease virus (IBDV), abbreviated as HVT-ND-IBD, will be used in chickens to induce protection against Marek's Disease (MD), Newcastle Disease (ND) and Infectious Bursal Disease (IBD). The backbone of this recombinant vaccine consists of the HVT virus FC126 which is a serotype 3 Marek's disease virus (MDV3) and nonpathogenic in avian species. The HVT backbone has been used in other HVT vectored vaccines which are on the market worldwide (e.g. Innovax-ILT, Innovax-ND, Vaxxitek HVT-IBD). Furthermore, the HVT FC126 virus has been used in the field as a vaccine to protect against Marek's Disease for over 40 years and has proven to be safe and efficacious. The HVT virus is a cell associated virus and therefore the vaccine consists of chicken embryo-fibroblast (CEF) cells infected with the HVT-ND-IBD virus and is presented as a cell suspension frozen and stored in liquid nitrogen.

Mode of action of the vaccine, use and relevance of the product:

After vaccination, the infected cells in the chickens express the F-protein of NDV and the VP2 protein of IBDV thereby eliciting an immune response directed against these proteins resulting in protective immunity against ND and IBD. The HVT virus induces protection against Marek's Disease.

Therefore, this vaccine will protect chickens against ND, IBD and MD with one vaccination that can be applied in the hatchery. Vaccination with HVT-ND-IBD provides lifelong immunity and eliminates field vaccinations with live NDV and IBDV vaccines.

The results of the proposed trial will generate field safety data and field efficacy data against ND, IBD and MD needed for licensure of the vaccine in Europe.

1.4. Geef een korte beschrijving van de voorgenomen werkzaamheden.

The planned activities in chronological order are:

1. Vaccination of the chickens with the HVT-ND-IBD vaccine via the in-ovo or subcutaneous route will be performed at the field location. Vaccinated chickens will be held for a duration of maximum 10 weeks.
2. A number of chickens will be transported to the animal facilities of MSD Animal Health in Boxmeer for efficacy studies. For activities with the recombinant vaccine at the Boxmeer site the contained use license IG98-085 applies.
3. Observation of the chickens. General performance and health status will be monitored for the duration of the study.

4. To monitor possible field infections with other pathogens, blood samples will be collected at the start, halfway and at the end of the trial.
5. Blood samples will be sent to the research laboratories of MSD Animal Health in Boxmeer for further processing (contained use license IG98-085).
6. All waste will be processed according to standard farm procedures as described in detail in section 4.9 of this application.

1.5. Beoogde begin- en einddatum:

Proposed starting date: 1 Jan 2016
Proposed end date: 31 Dec 2018

1.6. Wilt u andere informatie vertrouwelijk houden? Zo ja, geef een motivering die concreet aangeeft welke nadelige gevolgen openbaarmaking van deze informatie voor uw concurrentiepositie heeft.

Confidential information is provided in:
Appendix 01, 02, 03, 04, 05, 06, 07, 08, 09, 11, 12, 13 and 14 (appendices no. 10 and 15 are not confidential).

These documents contain detailed information about our product, which is commercial confidential and contains intellectual property/trade secrets. Making this information public will harm the competitiveness of our company.

Vergunningaanvrager

1.7. Naam rechtspersoon:

Intervet International B.V.

1.8. Adres rechtspersoon:

Wim de Körverstraat 35

1.9. Postcode en plaats van vestiging van de rechtspersoon:

5831 AN Boxmeer

1.10. Op welke locaties wordt de voorgenomen werkzaamheden uitgevoerd?

Location 1:
Conventional poultry farm (3 houses): one house will be included in the study (house 2).
Address: Kerkenhuisweg 3, 5441 PW Oeffelt

Location 2:
Conventional farm with poultry (4 houses) and cattle: one house will be included in the study (house 4)
Address: Thijsweg 2, 7122 KH Aalten

The supervision of the study is covered by appropriate provisions in the agreements between the farmers and veterinarians who are involved in the study.

2. Constructie en samenstelling van het GGO

Virus waarvan de genetisch gemodificeerde vector is afgeleid

2.1. Welk virus is gebruikt als uitgangsvirus bij de constructie van het GGO?

The HVT strain FC-126 is used for the construction of the recombinant virus. This strain was obtained from the American Type Culture Collection (FC-126 "Calnek", ATCC #584-C) bank. HVT or Meleagrid herpes virus 1 (MeHV-1) belongs to the family of Herpesviridae, subfamily of Alphaherpesvirinae, genus of Mardivirus and is a double stranded DNA virus of about 160kb. The HVT virus is a serotype 3 Marek's disease virus (MDV).

2.2. Beschrijf op welke wijze de identiteit van het uitgangsvirus bepaald is.

The history of the HVT FC-126 virus is indicated below in the information provided by the ATCC and in publications by Witter et al., 1970 and Okazaki et al., 1970.

Information provided by ATCC (VR-584C):

Strain:	FC-126 (Calnek).
Original source:	Blood of turkey without clinical symptoms.
References:	Witter, R.L. et al., Am. J. Vet. Res. 31:, 525, 1970 and Calnek, B.W. et al., Appl. Microbiol. 20: 723, 1970.
Preparation:	Extract of infected cells suspended in SPA (SPGA stabilizer but without sodium glutamate).
Host of Choice:	In vitro: CK; in vivo: chicken. Incubation: 5 days.
Effect:	in vitro: FFU and CPE; in vivo: nonpathogenic for chickens and turkeys. Protects against Marek's disease in chickens.
Host range:	In vitro: CK, duck EF, CEF; in vivo: turkey and chicken.
Special Characteristics:	Antigenically related to Marek's disease virus of chickens. When inoculated in 1-day-old chicks, will protect against MD when challenged subsequently with MDV.

Deposited and Prepared by: B.W. Calnek.
Shipped: Freeze-dried.

This virus was identified as described in the publication from Witter et al., 1970 and Okazaki et al., 1970. This HVT FC-126 virus was used to generate the overlapping clones which were used for the construction of HVT-ND-IBD. This cosmid set from HVT FC-126 was characterized by restriction analysis (BamHI). Restriction patterns were compared with the restriction profile of the HVT FC-126 sequence published in GenBank (accession number AF291866) and no differences were observed confirming virus identity.

2.3. Wat is het gastheerbereik van het uitgangsvirus?

Turkeys are the natural host of the parental HVT virus (Witter and Solomon, 1971). The virus is endemic in turkeys and turkey will get infected at early age with the HVT virus through inhalation of dust particles. The virus is host restricted and under normal conditions only turkeys will get infected by the natural route (inhalation). Infection of chickens by the natural route through inhalation has only been demonstrated under experimental conditions (Cho and Kenzy, 1975 and Cho, 1976). Chickens and some other non-target species like quails and pheasants can be infected experimentally, for example by injection. However, in all avian species the HVT virus is non-pathogenic (Witter et al., 1970, Marek's disease in Diseases of Poultry, 12th Ed. 2008)

The HVT virus, similar for all infected birds, is found in lymphoid cells (B- and T-lymphocytes) and in feather follicle epithelial cells (see scheme of HVT infection cycle presented in 2.6).

The HVT virus is used since 1972 as a vaccine for chickens against Marek's disease. Currently it is commonly used in combination with a serotype 1 MDV vaccine (Rispens) to protect against Marek's disease. The HVT vaccine has proven safe and efficacious. As a result of the widespread use of the HVT vaccine, the virus is ubiquitous in chickens (Marek's disease in Diseases of Poultry, 12th Ed. 2008).

2.4. Geef relevante gegevens over pathogeniciteit en eventuele attenuering en biologische inperking van het uitgangsvirus.

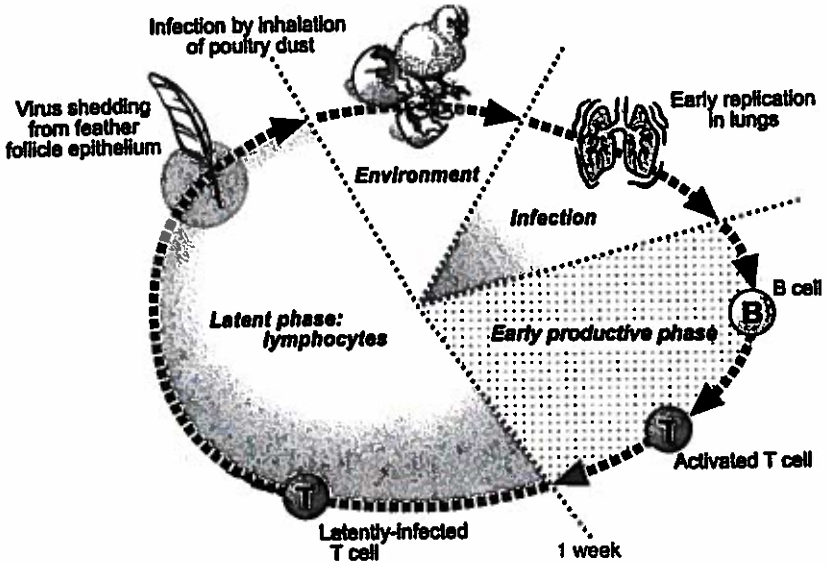
The HVT FC-126 virus is classified as a class 1 pathogen. It is a nonpathogenic virus in all avian species (Witter et., 1970, Marek's disease in Diseases of Poultry, 12th Ed. 2008) i.e.it has never been associated with any disease in chickens, turkeys or other poultry.

2.5. Welke ziekteverwekkende eigenschappen bezit het uitgangsvirus en welke behandelmethoden kunnen bij de bestrijding toegepast worden?

As indicated in section 2.4. the HVT FC-126 virus is nonpathogenic and will not cause any disease.

2.6. Via welke routes verspreidt het uitgangsvirus zich?

Turkeys are efficiently infected by the natural route but infection of chickens by the natural route is very rare and only demonstrated under experimental conditions (Cho and Kenzy, 1975 and Cho, 1976).
The different phases of infection are illustrated in the figure below. Natural infection of turkey occurs through inhalation of dust particles. Uptake of the virus is (presumably) by lung associated macrophages. Lymphoid cells become infected and get latently infected. In this phase the virus is cell-associated. In the final phase of infection feather follicle epithelial cells get infected and the virus will be shed, in a cell-free form, into the environment (Marek's disease in Diseases of Poultry, 12th Ed. 2008). Infectious virus will therefore be present in the dust and manure in the poultry houses.



Taken from Marek's disease in Diseases in Poultry, 12th Edition, editor in chief, Y.M. Saif, 2008. Figure is adapted to illustrate HVT infection in poultry via dust inhalation, replication in lymphoid cells, latency of the virus and shedding via feather follicle epithelial cells.

2.7. Hoe kan het uitgangsvirus buiten de gastheer overleven?

Survivability of the HVT virus in the environment has not been determined under field conditions but this has been determined for MDV serotypes 1 viruses. The stability of cell

associated virus is completely dependent on the viability of the cells. Any treatment affecting cell viability will decrease directly the infectivity of the virus.
Cell free MDV, released through shedding from feather follicle epithelial cells, present in dander, litter and manure is stable for much longer. At room temperature infectivity is retained for 4-8 months (Hlozanek et al., 1972, Witter et al., 1968). The virus is sensitive for high temperature and survival of virus may be affected reversely by increased humidity. HVT is inactivated by a variety of common chemical disinfectants within a 10-minute treatment period (Calnek and Hitchner, 1973, Hlozanek et al., 1977).

De genetisch gemodificeerde virale vector

2.8. Geef een beschrijving van de 'uitgangsvector(en)'.

The HVT FC-126 strain is used as vector backbone. For the construction of the recombinant viruses overlapping DNA fragments (cosmids) from the HVT FC-126 virus were used. This procedure has been described by van Zijl et al. (1988) for a different herpesvirus. To reconstruct the HVT virus the overlapping DNA fragments are transfected into cells. The different overlapping DNA fragments contain the complete information of the parent HVT FC-126 genome. This has been confirmed by restriction analysis, which shows that the overlapping fragments are in line with the published HVT FC-126 sequence in GenBank (accession no. AF291866; Afonso et al., 2001). One of these overlapping fragments was used to insert the expression cassette for the NDV F and the IBDV VP2 in order to generate the HVT-ND-IBD vaccine strain after homologous recombination in cells.
The same parental virus (cosmid library) was used for the generation of Innovax-ILT, where different genes were inserted (the Infectious laryngotracheitis virus gD and gI genes). This product has very recently been registered in EU.

2.9. Zijn er met betrekking tot de pathogeniciteit van het uitgangsvirus eigenschappen van de 'uitgangsvector' gewijzigd die bepalend zijn voor de pathogeniciteit van de uitgangsvector?

No adaptations to the parental HVT virus have been made. Characteristics of the HVT FC-126 have not been changed in any way, so the parental virus used for construction of the HVT-ND-IBD recombinant is nonpathogenic and is classified as a class I pathogen.

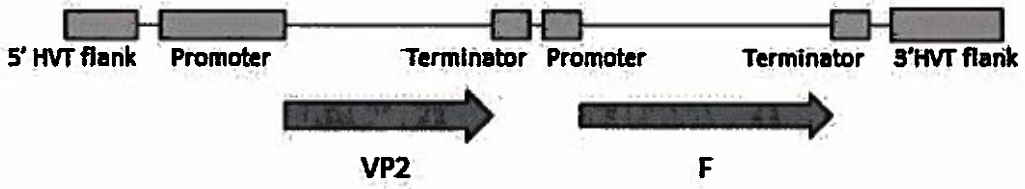
2.10. Geef een beschrijving van de wijze van vervaardiging van de virale vector vanuit de 'uitgangsvector(en)'.

See 2.8.
Briefly, for the construction of the recombinant viruses overlapping DNA fragments (cosmids) from the HVT FC-126 virus were used. To reconstruct the HVT virus the overlapping DNA fragments are transfected into cells. One of these overlapping fragments was used to insert the expression cassette for the NDV F and the IBDV VP2 in order to generate the HVT-ND-IBD vaccine strain after homologous recombination in cells.

2.11. Geef een beschrijving van de coderende genen en regulatoire sequenties die aanwezig zijn in de vector en in het in de vector geïnserteerde DNA.

Inserted genetic materials in the FC-126 backbone are the NDV F gene, the IBDV VP2 gene and regulatory sequences.

A schematic overview of the inserted genetic sequences in the HVT FC-126 genome is illustrated in the scheme below.



Schematic view of the inserted genetic material (insertion cassette) in the HVT FC-126 genome.

The donor organism of the VP2 gene is the IBD virus which is a member of the genus *Avibirnavirus*, family of *Birnaviridae*. It is a non-enveloped virus with a single-shelled icosahedral capsid and has a diameter between 55 and 60 nm. The genome consist of two double stranded RNA segments, designated A and B. Segment A contains two partly overlapping open reading frames (ORF) of which the largest one is autocatalytically cleaved into the structural proteins VP2 and VP3 and in the serine protease VP4. The virus is a non-enveloped virus with a capsid composed of trimers of VP2 that forms spikes projecting radially for the capsid. The VP2 capsid protein elicits neutralizing antibodies and represents the molecular basis for antigenicity (Ingrao et al., 2013). It has been demonstrated that a dominant fragment of VP2 can induce humoral and cellular immunity against IBD and elicits a protective immune response in chickens better that e.g. an attenuated viral strain and therefore VP2 (as a fragment) can be used as a vaccine or in a vaccine (Pradnan et al, 2012). This has e.g. been demonstrated for HVT-VP2 vaccines (with single insert; Ingrao et al., 2013). Expression of the VP2 protein by the HVT-ND-IBD recombinant will therefore results in a protective immune response against IBD.

The donor organism of the F-gene is the NDV which is a member of the genus *Avulavirus*, subfamily *Paramyxovirinae* and family of *Paramyxoviridae*. It is a pleomorphic enveloped, non-segmented, negative sense single stranded RNA virus of around 200-300nm. It encodes for 6 essential genes: the Nucleocapsid (N), matrix protein (M), phosphoprotein (P), fusion protein (F), haemagglutinin-neuramidase protein (HN) and the large polymerase protein (L). The F and HN proteins are the two surface glycoproteins that function as virus neutralizing antigens that are able to induce immunity. These proteins are responsible for virus attachment and fusion to the host cell membrane (Ganar et al., 2014). HVT vector vaccines with one inserted gene, like HVT-F, have been demonstrated to protect against ND in chickens (Morgan et al., 1993). Expression of the F-protein by the HVT-IBD-ND recombinant will results in protective immunity against ND.

Expression of the two inserted genes is regulated from heterologous viral promotors and terminator sequences from cytomegalovirus and Simian vacuolating virus 40. During the cloning of the promoter, terminator and the open reading frames remnants of cloning remain in the regions flanking the inserted elements. These remnant sequences have no function and no new open reading frames are created by these sequences.

The expression cassette was inserted in the US2 gene (HVT ORF088). This insertion site has been described in the literature by Gao et al (2011), where the effect of using the US2 gene as insertion site was evaluated. From these studies it was concluded that the plaque and growth kinetics of the HVT recombinant with the insertion in the US2 site was the same as the parental HVT. Furthermore, it was concluded that this insertion site was a favorable site for insertion of genes for generation of recombinant HVT vaccines. They demonstrated that after expression of the Influenza H5 gene from this insertion site good efficacy against H5N1 challenge was obtained. The exact function of US2 in HVT is not known. In MDV the US2 gene was shown to be non-essential for MDV growth in cell culture (Cantello et al., 1991). Studies performed with HVT-ND-IBD show good expression of the proteins, stability of the construct and efficacy against MD, ND and IBD, confirming the observations of Gao et al (2011) that the US2 site is a favorable site for insertion of genes.
The insertion of the expression cassette disrupts the US2 open reading frame: this results in a partial ORF at the 5'end of 373 nucleotides (124 amino acids), where the readings frame

is fused to 150 nt (50 amino acids) in the inserted region before a stop codon is present. Theoretically this partial ORF could be expressed. However, it is not expected to be functional.
The 3'end of the US2 ORF is not fused to other sequences. The first ATG in this sequence could result in a partial US protein over the last 348 nucleotide of the ORF (116 amino acids). Also this partial ORF, if expressed at all, is not expected to be functional.

2.12. Geef een moleculaire karakterisatie van de genetisch gemodificeerde virale vector.

Evaluation of HVT-ND-IBD was performed by sequence analysis of the insertion region. Sequencing confirmed that no changes had occurred in the expression cassette which was inserted in the genome. Approximately 400 bp of the HVT regions flanking the expression cassette have been determined by sequence analysis. No changes in the HVT sequence have been observed when a comparison was made using the parent HVT sequence; the parent sequence used is the sequence of the wild type HVT FC-126 virus that has been cloned and is used to assemble the HVT-ND-IBD from (sequence in homology vector). A comparison was also made to the HVT FC-126 sequence which has been deposited in GenBank (accession number AF291866). The 5' flanking region is 100% identical to the HVT sequence in GenBank and the 3' flanking region is 99.8% (472/473 nt) identical to the GenBank sequence. The single nucleotide mismatch is at a wobble position, and would not affect the protein sequence of the US2 ORF. This can be regarded as a natural polymorphism within strain HVT FC-126.

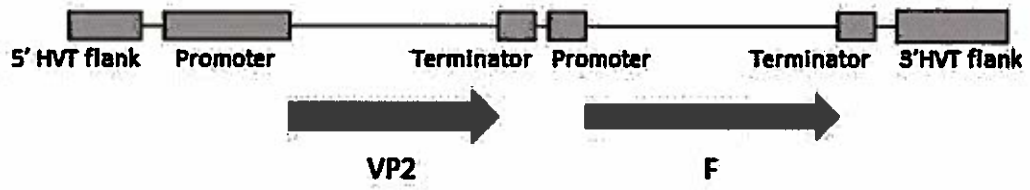
Southern blot analysis was performed using HVT-ND-IBD DNA to demonstrate that the expression cassette was correctly integrated into the genome. The evaluation showed that there was a single insertion of the cassette with the VP2 and F gene and the expression cassette is integrated in the correct orientation at the anticipated location in the HVT genome. Moreover, it was shown that there were no deletions, rearrangements or additional foreign sequences inserted in the HVT genome.

2.13. Beschrijf de herkomst van de cellen/cellijnen waarin de virale uitgangsvector wordt opgekweekt. Geef hierbij eveneens aan welke in de cellen aanwezige genetische componenten mogelijk aanleiding kunnen geven tot complementatie of recombinatie.

Chicken embryo fibroblasts (or CEF) are used to culture the HVT (recombinant) viruses. Cells are isolated from 10-11 day old specific pathogen free (SPF) chicken embryos and primary or secondary passages of these isolated cells (mainly fibroblasts) are used for culturing the HVT virus. It is very unlikely that the genetic material of these CEFs will interact with the genetic material from the parental HVT virus or the recombinant virus and recombination is very unlikely to occur as the avian and viral DNA do not show any resemblance with each other. To date, there is no evidence that HVT integrates into the genome of chickens or turkeys; HVT infection is not oncogenic, and studies with HVT have never revealed integration into the genome.
Furthermore, it can be noted that the chicken flocks used to produce the SPF eggs and embryos are regularly tested for the presence of viruses and bacteria. The chicken flocks are tested for: avian encephalomyelitis virus, avian infectious bronchitis virus, avian infectious laryngotracheitis virus, avian leucosis virus, avian nephritis virus, avian reovirus, avian reticuloendotheliosis virus, chicken anemia agent, avian adenovirus, infectious bursal disease virus, influenza A virus, Marek's disease virus, Newcastle disease virus, Turkey rhinotracheitis virus, Mycoplasma gallisecticum and synoviae, Salmonelle pullorum and SPP. No viral sequences will therefore be present in the CEF cells isolated from embryos that originate from these flocks and therefore the risk of recombination of viral sequences with the GMO during production is effectively zero.

2.14. Vat de gegevens samen in een schematische weergave ('kaart') van het genetisch gemodificeerde organisme. Geef hierbij eveneens de eventueel aanwezige relevante helpersequenties weer.

A schematic overview of the inserted genetic sequences into the HVT FC-126 genome is shown in the figure below. The VP2 gene of IBDV and the F-gene from NDV have been inserted in the HVT genome by homologous recombination. Expression of both genes is regulated by a promoter sequence and termination signals are added after both genes.



Schematic view of the inserted genetic material (insertion cassette) in the HVT FC-126 genome.

2.15. Geef aan in hoeverre het gastheerbereik van de genetisch gemodificeerde virale vector gewijzigd is of kan zijn ten opzichte van het uitgangsvirus.

Host range of the parental HVT virus and the recombinant virus are limited to avian species with turkeys as the natural host for the HVT virus. A study was performed to evaluate whether the insertion of the IBDV VP2 and NDV F-gene changed the properties of the HVT parent virus. The cell tropism in the chicken of the parental HVT virus and the HVT-ND-IBD recombinant virus was studied. It was found that both the parental HVT virus and the HVT recombinant replicate in lymphoid cells found in white blood cell suspension and tissues like the thymus, spleen and bursa and in feather follicle epithelial cells. No virus is found in the trachea. The results demonstrate that replication of the recombinant virus is comparable to that of the parental HVT virus in WBC, bursa and spleen at 1 to 5 weeks post infection. However, the amount of virus in the feather follicle epithelial cells was significantly reduced. The results demonstrate that only few virus particles are found of the recombinant virus in feather follicle epithelial cells suggesting that shedding of the recombinant virus will be much reduced to almost zero when compared to the parent HVT strain. Based on these results it can be concluded that the host range, and tissue and cell tropism from HVT-ND-IBD will not be any different compared to the parent HVT strain.
This is in line with what has been observed for other HVT recombinants, such as Innovax-ILT; also here the insertion of additional genes (gD and gI genes from Infectious laryngotracheitis virus, ILVT), did not change the tissue tropism, but did lead to a reduction of the amount of virus found in the feather follicles.

Regarding the host specificity of HVT viruses little is known about the specificity of the interaction of viral HVT proteins with their cognate cellular receptors. HVT viruses are cell associated virus (in the body) and only shed as cell free virus through the feather follicles. Natural infection is through inhalation (dust) and uptake of the virus by (presumably) lung associated macrophages. Little is known about this process but changes in host specificity would most likely be visualized by changes in cell tropism which is not the case (see next section).
Furthermore, it can be noted that shedding of the GMO is significantly reduced compared to the parent HVT virus.

2.16. Welke fysiologische (onder andere ziekteverwekkende) effecten kunnen door toedoen van de genetisch gemodificeerde virale vector ontstaan? Geef hierbij tevens aan welke behandelmethoden beschikbaar zijn.

The HVT recombinant vector will not affect the host in any other way than the parental HVT virus. No new physiological (or pathological) processes in the host will originate by the presence of the recombinant virus. As outlined above, the insertion of the IBDV VP2 and NDV F-genes did not change the tissue tropism. Furthermore, a contained use field trial was performed using the HVT-ND-IBD recombinant vaccine in UK. Chickens were vaccinated with HVT-ND-IBD and control chickens were injected with diluent by the subcutaneous route. Safety evaluations showed no differences between the vaccinated and control groups up to 21 days post vaccination. Similar mortality rates, feed intake, live weights, feed conversion rate (FCR) and the absence of clinical signs for birds in both groups indicates that vaccination of chickens with the HVT-ND-IBD vaccine was safe.
The safety of HVT-ND-IBD was also confirmed in turkeys: a more than 10 times dose of HVT-ND-IBD was administered to turkeys by s.c. injection. No clinical signs have been observed in these turkeys, confirming vaccine safety in turkeys.

2.17. Geef aan via welke routes de genetisch gemodificeerde virale vector verspreid kan worden.

HVT FC-126, which is the parental virus of the GMO is a fully nonpathogenic virus. Its natural host is the turkey but the virus can also infect and replicate in chickens. Infection of other avian species is very unlikely and can only occur by injection. HVT cannot infect humans. HVT causes no clinical disease in turkeys and chickens. In turkeys the virus can spread via inhalation of dust particles shed from the skin from infected (or vaccinated) birds. Spreading of the virus to chickens via the natural route (inhalation of dust particles) is highly unlikely as it is only demonstrated under experimental conditions. Shedding however is observed in (vaccinated) chickens although limited and transient in nature.
Genetic modifications made by introducing the F-gene and VP2 gene did not change the phenotype of the parent virus and the recombinant is therefore still fully nonpathogenic, similar to other registered HVT recombinants. The dissemination study demonstrated that the tissue tropism was still the same, but the amount of virus detected in the feather follicles was significantly reduced in the GGO vaccinated birds. This demonstrates that the insertion of the IBDV VP2 and NDV F-genes have reduced the shedding of the virus from vaccinated chickens. Although the spreading from HVT-ND-IBD is reduced, it cannot be excluded the GGO can spread to turkey and therefore no turkeys are allowed near the site.

3. Productie van het GGO

3.1. Geef aan onder welke verantwoordelijkheid productie van het GGO wordt uitgevoerd.

Antwoord:

O Productie wordt uitgevoerd onder verantwoordelijkheid van de aanvrager en maakt deel uit van deze vergunningaanvraag.

● Productie wordt uitgevoerd door en onder verantwoordelijkheid van de aanvrager maar maakt geen deel uit van deze vergunningaanvraag:
  - O Voor de productie zal een aparte aanvraag onder ingeperkt gebruik worden ingediend
  - O Voor de productie wordt verwezen naar een reeds bestaande Ingeperkt Gebruik vergunning:
    (Geef hierbij het nummer van de betreffende GGO vergunning)
  - ● De productie wordt uitgevoerd onder verantwoordelijkheid van derden. Indien de productie plaatsvindt in Nederland wordt u verzocht het nummer van de betreffende GGO vergunning te vermelden. Wanneer de productie plaatsvindt buiten Nederland gelieve u dit aan te geven.

Production will take place at Merck/MSD Animal Health in de Bilt (NL). The GMO license for production in the Bilt is IG13-012.

3.2. In welke stappen van de productie vindt kwaliteitscontrole plaats, welke testmethoden worden gebruikt en hoe worden de tests uitgevoerd.

The vaccine will be produced on CEF cells. The production process and controls in place are similar to other Marek vaccines produced by Merck/MSD. CEF cells are infected with vaccine virus, harvested, formulated in freezing medium and frozen in glass vials in liquid nitrogen. The in-process-control tests that are performed determine the presence of CPE (cytopathic effect) and the filling volume of the vials. CPE evaluation is done by microscopic evaluation of the cells. The filling volume is checked by weighing.

3.3. Welke criteria worden aan een batch van het GGO gesteld voordat deze wordt vrijgegeven voor de onderhavige toepassing.

A batch of the vaccine will be released according to European monograph 0589 (Marek's disease vaccine (live)). This means that every batch of vaccine will be tested for identity, sterility (bacteria and fungi), mycoplasma, extraneous agents and virus titer:

Identity testing:
Identity of the virus is confirmed by immuno-fluorescence staining of virus plaques using a specific antibody for the VP2 or the F gene.
For release of the vaccine the identity should be confirmed by a positive staining.

Sterility testing (bacteria and fungi):
According to Ph.Eur. 2.6.1 and monograph 0062. Copies of the monograph are provided in appendix 16. Test methods and detection limit in line with EU requirements are described in these monographs.
For release of the vaccine there should be no growth.

Mycoplasma testing:
According to Ph.Eur. 2.6.7. A copy of the monograph is provided in appendix 16. Test methods and detection limit in line with EU requirements are described in detail in this monograph.
For release of the vaccine there should be no growth.

Extraneous agents testing:
For extraneous agents testing the vaccine will be analyzed for the presence of egg drop syndrome (EDS) virus, turkey rhinotracheitis virus, chickens anemia virus, duck enteritis virus, duck and goose parvovirus and viral agents that can grow in embryonated chicken eggs or on chicken embryo fibroblasts.
The extraneous agents testing is performed according to Ph.Eur. 2.6.25. A copy of the monograph is provided in appendix 16. Test methods and detection limit in line with EU requirements are described in detail in this monograph.
For release of the vaccine, there should be no extraneous agents detected.

Virus titer:
The virus titer is determined by a standard plaque assay on CEF cells. Serial dilutions are prepared and incubated on CEF cells. At appropriate dilutions the HVT-ND-IBD virus plaques are visualized using immune-fluorescence and the plaque forming units (PFU) are counted and the virus titer is calculated.
For release of the vaccine, there is no specific requirement, but the virus titer is needed to determine the dose of the vaccine to be used in the field trial.

Further details on the batch testing and release are also provided in the European monograph 0589; Marek's disease vaccine live (Appendix 15).

4. Beschrijving van het onderzoek

4.1. Hoeveel proefdieren zullen deelnemen aan het onderzoek?

In total a maximum of 60.000 chicks/eggs will be included in the study. This number takes into account the flock sizes in the field (10.000-12.000 chickens per flock/group taking into account the size (m2) of the stables). A possible repetition of the study is included in the number.

4.2. Beschrijf op welke wijze de batch met het GGO getransporteerd en gereedgemaakt wordt voor toediening aan het proefdier.

The vaccine will be transported in liquid nitrogen to the poultry farm. The vaccine will be prepared in a separate room on the poultry farm. The ampoules are removed from the nitrogen container and thawed. The contents of the ampoules will be diluted into the correct amount of solvent according to standard procedures (for detailed description of vaccine preparation see the leaflet of Innovax-ILT).

Details on vaccine preparation are provided in Appendix 10 (leaflet of Innovax-ILT).

4.3. Op welke wijze wordt het GGO preparaat aan het proefdier toegediend?

The vaccine will be administered at the farm to day-old chicks by subcutaneous injection in the neck or *in-ovo* to 18-day embryonated eggs using an in-ovo vaccinator. In the latter case these eggs will be delivered to the farm where they will be vaccinated and hatch a few days later. For both vaccination methods standard commercial equipment and techniques will be used.

In-ovo vaccination
For *in ovo* vaccination 18 day embryonated eggs are injected with the vaccine using the in-ovo vaccination machinery. Eggs are cleaned and a small hole is made in each egg. Next, a needle goes through these holes and the vaccine is injected into the egg. After injection eggs are placed in the hatching incubator. The eggs will hatch after a total of 21 days of incubation.

Subcutaneous vaccination
For subcutaneous vaccination one day old chickens are injected with the vaccine under the skin in the neck.

Details on precaution for use of the vaccine are provided in Appendix 10 (leaflet of Innovax-ILT).
The precautions taken to prevent contaminating of hands, eyes and clothing with the vaccine suspension are wearing gloves, safety goggles and a coverall during vaccine preparation and administration. These are general precautions which are taken when working with biological materials such as vaccines.

4.4. Welke doses worden toegediend en op welke tijdstippen gedurende de studie vindt toediening plaats?

Vaccination will be once with one field dose of HVT-ND-IBD. One field dose of HVT-ND-IBD will contain between $10^{3.0}$ and $10^{4.6}$pfu/dose.

4.5. Welke andere medicatie wordt aan het proefdier toegediend die mogelijk van invloed kan zijn op het toe te dienen GGO preparaat?

The animals in the study will be routinely vaccinated twice against infectious bronchitis (IB) with a live vaccine at one day of age and between 14-18 days of age. The live infectious bronchitis (IB) vaccines used for vaccination are conventional live attenuated vaccines. IB is

a common infection of poultry and practically all commercial chickens in the Netherlands are routinely vaccinated with conventional live IB vaccines, which are generally administered by spray.
Concurrent administration of a live IB vaccine (by spray) and a Marek's disease vaccine including HVT FC-126 vaccines is common practice in the field. No interactions between IB vaccines and Marek's disease vaccines have been demonstrated and it is therefore unlikely that the GMO vaccine and IB vaccine will interact or influence each other.

The risk of recombination of IB vaccines and the GMO is effectively zero as no homologous sequences are found in both viruses. IB viruses belong to the genus of *gammacoronavirus* and the GMO to the genus of *mardivirus* (family of *alphaherpesviridae*; HVT) containing sequences of avibirnavirus genus (VP2 gene) and *avulavirus* genus (family of *paramyxoviridae*; F-gene).

Bemonstering

4.6. Beschrijf welke monsters van het proefdier worden genomen die GGO's (kunnen) bevatten?

Blood samples will be collected at vaccination, half-way during the study and at the end of the study. The blood samples taken during and at end of study may contain the GMO (lymphocytes).

Blood sampling at one day of age will be done at MSD Animal Health in Boxmeer. These activities are included in permit IG 98-085.

At both farms blood sampling will be done at approximately 21 days of age (half way the study) and at the end of the study when the birds are max. 10 weeks of age.

Study with in-*ovo* vaccination:

Time point	Activity	Time between vaccination and sampling
Day -3 (In-*ovo* vaccination)	In-*ovo* vaccination of 18 day embryonated eggs	n/a
Day 21	Blood sampling	24 days
Before euthanasia	Blood sampling	Max 10 weeks

Study with subcutaneous vaccination:

Time point	Activity	Time between vaccination and sampling
Day 0 (day of arrival of day-old chicks)	Subcutaneous vaccination (subcutaneous)	n/a
Day 21	Blood sampling	21 days
Before euthanasia	Blood sampling	Max 10 weeks

4.7. Beschrijf hoe bemonstering plaatsvindt en hoe de monsters verder worden verwerkt.

Blood will be collected from the vein in the neck or wing (by making a small incision in the wing vein). Contamination of the GMO in the stable can only take place in case blood is spilled during blood collection. The GMO which is present in the lymphocytes is however not able to spread, because these cells are not viable outside the body.

The blood samples will be transported in bio-containers from the poultry farm to MSD Animal Health according to bijlage 1 van de Regeling GGO 2013.
Processing of blood samples will take place in the laboratory of MSD Animal Health in Boxmeer. These activities are included in permit IG 98-085 (and are therefore not part of this application).

4.8. Op welke wijze wordt het GGO preparaat gedetecteerd na de toediening?

The presence of the GMO in the vaccinated chicken will not be investigated. Immunity as a result of GMO presence will be confirmed by efficacy testing.

Afvalverwerking

4.9. Geef een overzicht van de aard en hoeveelheid van het geproduceerde afval en beschrijf hoe het afval wordt afgevoerd.

The nature, amount and the way of disposal of waste produced during the complete trial such as manure, litter and wastewater will not change by the application of the GMO compared to normal husbandry at the farm. The litter and manure from the stables with the HVT-ND-IBD vaccinated chickens will be transported to a manure combustion plant, where it will be destroyed by incineration. This material will be transported to the combustion plant in closed containers.
The chicken houses will be cleaned and disinfected with a disinfectant, effective against microbiological agents including viruses as commonly used by poultry farms, after disposal of the manure and litter. The wastewater which is produced by cleaning the stables at the end of the study will be processed according to the standard practice on the farm: the waste water will be collected in the manure pit and then distributed (if desired) over the land.
All vaccinated chickens that die or will be euthanized during or at the end of the study will be presented for destruction at a rendering plant (Cat. 1 material for disposal at Rendac). Materials such as needles, empty bottles, etc. will be disposed as clinical waste.

5. Milieurisicobeoordeling

Milieugerelateerde gegevens afkomstig uit eerdere experimenten

5.1. Geef een beschrijving van de resultaten welke afkomstig zijn uit eerdere studies met het GGO, en die van belang zijn voor de milieurisicobeoordeling.

In the studies indicated below dissemination and field safety data of the HVT-ND-IBD are presented as well as safety studies from another HVT vector vaccine, Innovax-ILT, which is comparable to HVT-ND-IBD in that the same parent strain was used for insertion of the ILT genes.

In these studies the safety of HVT recombinant vectors is investigated and the characteristics (phenotypic) compared with their parental virus HVT FC-126. All studies demonstrate that HVT recombinant vectors behave similarly as their parental virus HVT FC-126 indicating that insertion of genes (whether the VP2 and F genes in the HVT-ND-IBD recombinant virus, or the gD and gI genes in Innovax-ILT) do not change the behavior of the virus. The HVT FC-126 virus is a serotype 3 Marek's disease (MD) virus with the turkey as natural host. The HVT FC-126 virus is endemic in turkeys and widespread around the world. Lymphoid cells are the target cells of the virus but the virus is not cytolytic and does not cause lesions to thymus, bursa and spleen. The virus is excreted through the feather follicles and spreads through dust and manure in poultry houses. The virus is also not oncogenic (whereas serotype 1 MD virus is) and causes no clinical signs of disease. It also does not have impact on the immune system. In conclusion the virus is nonpathogenic in turkeys, chicken and other poultry species (Marek's disease in Diseases of Poultry, 12th Ed. 2008).

Dissemination studies in comparison to the parent virus

A dissemination study was performed with HVT-ND-IBD to evaluate whether the insertion of the IBDV VP2 and NDV F-gene changed the properties of the HVT parent virus. The cell/tissue tropism in the chicken of the parental HVT FC-126 virus and the HVT-ND-IBD virus was compared. Chickens were vaccinated with an overdose of HVT-ND-IBD and HVT FC-126 by the *in ovo* route (50 eggs were used) and 8, 15, 22, 29 and 36 days post inoculation white blood cells (WBC), spleens, bursa's, tracheas and feathers tips were collected from the vaccinated chickens and analyzed for the presence of virus by titration (detection of plaque forming units (PFU)). The results showed that both the parental HVT virus and HVT-ND-IBD replicate in lymphoid cells found in white blood cell suspension and tissues like the thymus, spleen and bursa and in feather follicle epithelial cells. No virus is found in the trachea. The results demonstrate that replication of the recombinant virus is comparable to that of the parental HVT virus in WBC, bursa and spleen at 1 to 5 weeks post infection. However, the amount of virus in the feather follicle epithelial cells was significantly reduced. The results demonstrate that only few virus particles are found of the HVT-ND-IBD virus in feather follicle epithelial cells indicating that shedding of HVT-ND-IBD will be much reduced (to almost zero) when compared to the parent HVT strain. Based on these results it can be concluded that the host range, and tissue and cell tropism from HVT-ND-IBD are not different compared to the parent HVT strain and the shedding and spreading potential is reduced.

Also for Innovax-ILT a similar study was performed to compare the tissue tropism of Innovax-ILT and HVT FC-126 in chickens. The set-up of this study was identical to the study with HVT-ND-IBD described above: chickens were vaccinated with an overdose of the Innovax-ILT vaccine by subcutaneous vaccination. At 8, 15, 22, 29 and 36 days post inoculation white blood cells (WBC), spleens, bursa's, tracheas and feathers tips were collected from the vaccinated chickens and analyzed for the presence of virus by titration (detection of plaque forming units (PFU)). Also here it was found that both the parental and

recombinant virus replicated in lymphoid cells present in the WBC, spleen and bursa and in the feather follicles epithelial cells and also no virus was found in the trachea. Also the Innovax-ILT vaccine strain replicated at a significantly lower level in the feather follicles

It can be concluded that for both HVT recombinants the insertion of the genes, did not change the pattern of virus distribution (tissue tropism) compared to the parent HVT FC-126. Furthermore, some quantitative differences were observed between HVT FC-126 and Innovax-ILT and HVT-ND-IBD in that the level of replication in the feather follicles was significantly reduced. This was most evident for HVT-ND-IBD as only a few virus particles could be detected.
Based on these results it can be concluded that chronology of virus appearance and tissue tropism of Innovax-ILT and HVT-ND-IBD is not any different from to the parent HVT strain but the shedding is reduced. These studies are relevant for this application as they demonstrate that insertion of genes does not change the properties of the parental HVT virus which has been used as a safe vaccine for decades. Also, the demonstration that the shedding of the HVT viruses is significantly reduced (almost no shedding for HVT-ND-IBD), further lowers the possibility for dissemination in the environment when used in the field trial.

Interaction studies with other viruses
Besides expressing the NDV F gene and the IBDV VP2 gene, the HVT-ND-IBD has not acquired biological properties that are not already present in the parent HVT FC-126 (see dissemination study above). Despite decades of intense poultry husbandry and co-infection of commercial poultry with MDV1, MDV2 and HVT, no data has been found of interspecific recombination among these avian herpesviruses or other viruses. Therefore recombination with other viruses is not expected to occur in the field.

For the Innovax-ILT vaccine a safety study was performed where a 10 times higher dose than standard of Innovax-ILT was given simultaneously with a 10 times higher dose of MDV serotype 1 vaccine (Nobilis Rismavac, which contains the Rispens strain). The birds were monitored for 120 days post vaccination for general appearance and health and for specific clinical signs associated with Marek's disease (MD). At the end of the study (day 120) all birds were euthanized and examination was performed with a focus on macroscopic lesions of Marek's disease (MD) e.g. atrophic bursa and thymus, visceral tumors and nerve lesions. In this study control chickens were found to be fully susceptible to the virulent challenge virus. An overdose of Innovax-ILT with or without MDV serotype 1 vaccine was safe in chicken as the performance of the chickens was good and no notable clinical signs or macroscopic lesions of Marek's disease were observed and no chickens died from causes attributable to the vaccine.
It can be concluded from this study that Innovax-ILT is safe in chickens both when used alone or simultaneously with another MDV serotype 1 strain (Rispens).

Field safety
A contained use field trail was performed using the HVT-ND-IBD vaccine in UK. 2205 chickens were vaccinated with HVT-ND-IBD and 2231 control chickens were injected with diluent by the subcutaneous route. Safety evaluations showed no differences between the vaccinated and control groups up to 21 days post vaccination. Similar mortality rates, feed intake, live weights and feed conversion rate (FCR) and the absence of clinical signs for birds in both groups indicates that vaccination of broiler chickens with the HVT-ND-IBD vaccine was safe.

For Innovax-ILT two field safety studies were performed investigating the safety of the vaccine (mixed with MDV serotype 1 vaccine, Rismavac); the first trial was performed in the Netherlands and the second trial in the US.

In a poultry rearing farm in the Netherlands a blinded field trial with matched controls (i.e. within a house divided into two almost identical compartments using birds from the same

parent flock, hatched at the same time, in the same hatchery) was performed. Day-of-hatch chicks of the test group were vaccinated subcutaneously at the farm with a standard dose of Innovax-ILT mixed with a standard dose of Nobilis Rismavac and the birds of the control group were vaccinated subcutaneously with a standard dose of Nobilis Rismavac+CA126. The safety was assessed on basis of mortality (daily observations for the first three weeks and weekly observations during the remainder of the study), morbidity (daily observations for the first three weeks and weekly observation during the remainder of the study), local reactions (evaluated at 1 day, 1 week, 2 weeks and 3 weeks post vaccination) and body weight gain (weight was taken at the end of the trial) and the study ended 12 weeks after vaccination.
In the first week, mortality was 0.27% (test) and 0.48% (control) only, which is very low. During the rest of the trial period mortality was negligible except on Day 19 when 20 animals accidentally became crushed by the feeding line. No difference in mortality between both groups was observed. No difference in morbidity between both groups was observed and in none of the 25 birds checked per group local reactions were observed after the vaccination. Only a slight difference in mean body weight between the two groups was observed, the weight of the birds vaccinated with Innovax-ILT mixed with Nobilis Rismavac being 30 grams heavier than those vaccinated with Nobilis Rismavac+CA126.
In conclusion, subcutaneous vaccination with Innovax-ILT mixed with Nobilis Rismavac is safe for day-of-hatch chickens.

In the poultry rearing farm in the USA a large group of day-of-hatch chicks were vaccinated subcutaneously at the hatchery with a standard dose of Innovax-ILT plus Rismavac (test group) or subcutaneously with a standard dose of Rismavac+FC-126 vaccine. The controls were vaccinated later during rearing against ILT. At the end of the rearing period (16 weeks of age) 16,128 birds per group were transported to the production farm. The egg production was recorded through 60 weeks after vaccination. The safety was primarily assessed on the basis of daily clinical observations and mortality by the farm staff for vaccination reactions during the first 3 weeks, and then by weekly mortality and morbidity thereafter. The study ended 60 weeks after vaccination.
In the first week mortality was very low and within normal range under these management conditions. During the entire period of pullet rearing (week 0-16) mortality rates between both groups were very similar (0.50% difference) and during the 45 weeks of the layer phase mortality rates were low and essentially the same between the two groups. No differences in morbidity between the two study groups were observed during the study. Moreover, in none of the birds checked at 1 day, 7 days, 15 days and 21 days after vaccination during the first 3 weeks any local reactions were observed after the vaccination. Egg production monitoring started at week 19 and was followed through week 60. There was no difference in egg lay between the control and test groups.
In conclusion, subcutaneous vaccination with Innovax-ILT mixed with Rismavac is safe for day-of-hatch chickens.

The contained use field trial with HVT-ND-IBD is relevant for this field trial application, as the safety of the vaccine for chickens is already shown in this field trial. More extensive field trials have already been performed with Innovax-ILT, demonstrating that HVT recombinant vaccines with new genes inserted is safe for chickens.

Furthermore, it can be noted that the Innovax-ILT vaccine is already on the market outside the EU and has been safety used since 2007 (the Innovax-ILT vaccine was registered in Europe recently and will be introduced in the market this year). The same is true for other HVT recombinant vaccines such as Innovax-ND (HVT with the same NDV F gene inserted as present in HVT-ND-IBD).

Conclusion:
Based on the studies performed and experience of use of these vaccines in the field, it can be concluded that HVT vaccines with genes of other viruses inserted, such as Innovax-ILT, Innovax-ND and the new HVT-ND-IBD V are safe vaccines for use in poultry.

Risicobeoordeling

5.2. Geef aan welke mogelijke nadelige effecten gepaard kunnen gaan met blootstelling van mens of milieu aan het GGO.

Assessment of risk

a) Pathogenicity or other adverse effects.
HVT is naturally nonpathogenic and its host range is restricted to avian species. A study was performed to study the replication and dissemination of the HVT-ND-IBD virus in vaccinated chickens (in comparison to the parent HVT FC-126 vaccine strain), demonstrating that the insertion of the NDV F gene and the IBDV VP2 gene have not changed the safety profile of the virus. This has been confirmed by other HVT recombinants which are used worldwide:
- Innovax-ND, has the same NDV F gene inserted and has been used since 2007 in the US and other countries worldwide.
- Vaxxitek HVT-IBD, also has the IBDV VP2 gene inserted and was licensed in the EU in 2002.
The safety of the HVT-ND-IBD vaccine was further confirmed in a contained use field trial in the UK.
Therefore, it can be concluded that HVT-ND-IBD is nonpathogenic for avian species and the safety is identical to the classical HVT vaccines, which have been safely used in the poultry industry for over 40 years now.

b) Genetic instability/gene transfer
HVT-ND-IBD is both phenotypic and genotypic stable. The GMO is based on a natural nonpathogenic HVT strain where NDV F gene and the IBDV VP2 gene have been inserted. No attenuation of the strain was done.
The stable insertion of the genes in the HVT FC-126 recipient when passaged *in vitro* has been demonstrated. Genetic stability of the construct was studied by passaging in CEF cells up to passage level 5. The genetic stability was demonstrated by sequence analysis of the insertion site and flanking regions.
Mutations in the sequence of HVT-ND-IBD are not more likely to occur compared to the parent HVT FC-126 which is established as a very safe vaccine. In case the gene insert is lost, this will not affect the nonpathogenic nature of the virus (the strain would become a normal HVT which in itself is fully nonpathogenic).
Phenotypic stability was also demonstrated. Expression of the NDV F gene and the IBDV VP2 gene was demonstrated by immunofluorescence staining on infected cells using specific antibodies and a monoclonal antibody for HVT. Staining with F or VP2 antibodies, demonstrated that there was a 100% expression of both genes in all HVT-ND-IBD plaques analyzed when infected with virus batches at up to passage level 15.

c) Capacity to survive, disseminate and establish
Since HVT-ND-IBD is a virus, it cannot replicate outside the animal. HVT-ND-IBD is a cell-associated vaccine and can only be infective as long as the cell remains viable. Many relatively gentle treatments like heating or freezing, or even prolonged incubation at room temperature will therefore destroy infectivity. Therefore, it can be concluded that the vaccine preparation itself cannot survive, establish and disseminate in the environment.

Survival and dispersal into the environment can only occur after vaccination through virus present in feather follicle epithelial cells from vaccinated chickens. From the feather follicles virus can be released that becomes part of dust particles. Stability of HVT in dust particles has not been determined under field conditions, but Marek's disease virus (MDV) in feather dust has been shown to be stable at least for several months at room temperature.

Replication of HVT-ND-IBD is comparable to that of the parental HVT virus in WBC, bursa (and spleen) at 1 to 5 weeks post infection but not in feather follicle epithelial cells. The results demonstrate that only few virus particles are found of HVT-ND-IBD in feather follicle epithelial cells suggesting that shedding of HVT-ND-IBD will also be less (to almost zero) (see 5.2.1.c). Nevertheless, like for HVT it should be assumed that vaccinated chickens can shed the vaccine virus during their life time.

In order to replicate, virus shed from the feather follicles will have to re-infect a permissive host which is described below under d).

d) Capacity to transmit to target and non-target species
HVT FC-126, which is the parental virus of the GMO is a fully nonpathogenic virus. Its natural host is the turkey. Infection of other avian species is very unlikely and only occasionally observed when the virus was inoculated directly by injection. HVT cannot infect humans. HVT causes no clinical disease in turkeys and chickens. In turkeys the virus can spread via inhalation of dust particles shed from the skin from infected (or vaccinated) birds. Spreading of the virus to chickens via the natural route (inhalation of dust particles) has not been demonstrated under field conditions. Shedding however is observed in (vaccinated) chickens although limited and transient in nature.
Genetic modifications made by introducing the NDV F gene and IBDV VP2 gene did not change the phenotype of the parent virus and the recombinant is therefore still fully nonpathogenic, similar to other registered HVT recombinants.
After injection, the vaccine virus may therefore spread from turkeys (its natural host) to turkeys and from chickens to turkeys, however, in both occasions already at a very low level. Some non-target species like quails and pheasants might be infected by injection but in these species the HVT vaccines cannot spread to birds in direct contact.

e) Potential for gene transfer
Genetic transfer to an organism in the environment has never been described for herpesviruses and is therefore unlikely to occur. The stable insertion of the NDV F gene and the IBDV VP2 gene in the HVT FC-126 recipient will not change this property. In the field trial vaccination is also performed with live infectious bronchitis (IB) vaccines. The risk of recombination of IB vaccines and the GMO is effectively zero as no homologous sequences are found in both viruses.

f) Products of expression of inserted sequences
The expressed NDV F gene and the IBDV VP2 gene are not harmful. Upon expression, they induce an immune response to NDV and IBDV in the vaccinated chickens.

g) Pathogenicity to other organisms
As indicated under 1.a above the host range of HVT-ND-IBD is limited to avian species and the virus does not replicate in mammals. Some non-target species like quails and pheasants can be infected by injection but in that case the vaccine virus cannot spread to birds in direct contact.

h) Potential for other effects
HVT-ND-IBD is grown in chicken embryo fibroblast (CEF). These are derived from specific pathogen free chicken flocks, which are monitored for the absence of viruses. No viral sequences will therefore be present in the CEF cells and therefore the risk of recombination of viral sequences with the GMO during vaccine production is effectively zero.
The HVT backbone of HVT-ND-IBD has been modified by the insertion of a fragment containing the NDV F gene, the IBDV VP2 gene, promoter and terminator sequences. Also some remnants of cloning are present in the inserted fragment (non-coding sequences). The insertion site is in the US2 gene of the HVT genome and this gene is deleted by the insertion. Remnants of the US2 gene could theoretically be expressed, but these partial proteins, if expressed at all are no longer functional.

A study was performed, demonstrating that the phenotype of this vaccine strain is the same as that of the HVT FC-126 parent. Therefore it can be concluded that the use of indicated promoter and terminator sequences and the presence of remnant sequences will have no impact on the characteristics of the GMO and therefore the likelihood that the GMO will acquire properties that render it less safe than the parental HVT FC126 virus is effectively zero. In addition, the presence of these inserted sequences will not increase the risk of recombination with other viral sequences or the chicken genome and therefore the likelihood that the GMO will transfer genes is effectively zero.
If the inserted genes would be lost, this would result in the wild type HVT, which is also fully nonpathogenic. Hence, there is no potential for other effects of the live vaccine strain.

In conclusion: HVT-ND-IBD cannot establish in the environment and poses no threat to other species.

5.3. Geef aan volgens welk scenario het GGO zich vanuit het proefdier kan verspreiden in het milieu.

Assessment of risk

a) Potential for exposure to the GMO
(i) Type of packaging and administration
The vaccine is presented deep frozen in sealed ampoules and only the number of ampoules required for vaccination is thawed and opened. During dilution of the vaccine virus suspension into diluent vaccination personnel or environment may come into contact with the vaccine strain.
Also the vaccine is contained in glass ampoules stored in liquid nitrogen and there are reports that ampoules might explode when removed from the cold storage and thawed. This also may expose the skin of the user to the vaccine or result in damage from the glass.
After a vaccine spill, the cell-associated vaccine virus will not be able to survive.
The probability of exposure is moderate. As the GMO cannot infect humans the consequences of exposure are negligible and therefore the risk is effectively zero.

(ii) Route of administration
The vaccine is administered by injection subcutaneously in the neck of one day old chickens and the person performing the injection will normally not be exposed to the vaccine strain. However, self-injection may accidently occur.
When the vaccine is applied *in ovo*, using *in ovo* vaccination equipment.
The probability of exposure is low. As the GMO cannot infect humans the consequences of exposure are negligible and therefore the risk is effectively zero.

(iii) Shedding of live product organism
Similar to HVT, HVT-ND-IBD can be shed from vaccinated birds from the feather follicle epithelium, although at a very low level compared to the parent virus. Nevertheless, like for HVT it must be assumed that vaccinated chickens can shed the vaccine virus until time of slaughter. Personnel on the farms or veterinarians may come into contact with the vaccine strain by contact to or inhalation of dust particles in the chicken houses.
The probability of exposure is moderate. As the GMO cannot infect humans the consequences of exposure are negligible and therefore the risk is effectively zero.

The HVT virus can spread poorly to other chickens in direct contact with vaccinated chickens and therefore the likelihood that this will happen is low.
The HVT virus can spread to turkeys in direct contact with vaccinated chickens and via the natural route (via inhalation of feather follicle dust). The chances of turkeys coming in contact with vaccinated chickens are very low, as the locations chosen will not have turkeys in the neighborhood and therefore the likelihood of this happening could be regarded as very low.

In the unlikely event of a turkey being infected with the vaccine virus, the turkey could transfer the virus to a contact turkey and spread the vaccine virus. But taking into consideration that HVT is endemic and ubiquitous in domestic turkeys and shedding of the HVT-ND-IBD virus is less compared with the parental HVT FC-126 strain, the likelihood of spread of the vaccine virus in turkeys is very low.

Overall the probability for exposure is moderate; however, this will not have adverse effect or result in spreading to the environment.

5.4. Geef een inschatting van de kans dat de in 5.2 beschreven nadelige effecten ook daadwerkelijk kunnen optreden.

Assessment of the likelihood of exposure

As described above the likelihood of skin exposure to the vaccine is moderate. The consequences of skin exposure to the vaccine are considered to be negligible. For vaccination of ~10.000 chickens, 10 ampoules of vaccine (1000 dose/ampoule) will be used. The overall risk of skin exposure of the vaccine to the user is therefore effectively zero.
The chance of accidental self-injection of the vaccine to the user is low. The consequence of accidental self-administration is negligible. The overall risk of accidental self-administration to the user is therefore considered to be effectively zero.
As described above the chance of exposure to dust particles containing the vaccine is moderate. The consequences of this exposure to the vaccine are considered to be negligible, as the GMO cannot infect humans and therefore the risk is effectively zero.

As described above the likelihood of spread of the virus to turkeys is very low. The consequences of spread to turkeys or other avian species are negligible. So, taking all the risk factors in consideration, the assessment of the level of risk to the environment for HVT-ND-IBD can be considered as effectively zero.
It should furthermore be noted that several different HVT recombinant vaccines are already on the market inside and outside the EU since 2002. The applicant has since 2007 two HVT recombinant HVT vaccines on the market, Innovax-ILT and Innovax-ND, and no adverse reactions associated with either HVT recombinant have been reported.

5.5. Beschrijf de risico's die op kunnen treden ten gevolge van de toepassing van het GGO, waarbij de effecten van eventuele risicomanagementmaatregelen zijn meegenomen.

1. Assessment of risk to humans

HVT can only infect avian species and does not infect humans. Studies were performed to show that the insertion of the NDV F gene and the IBDV VP2 gene did not alter the biological properties of the strain. Therefore no replication of HVT-ND-IBD in humans (healthy or immunocompromised) can be expected. The GMO does not possess any incorporated antibiotics resistance genes or other markers with potential hazard for humans.

Skin contact: As HVT-ND-IBD cannot infect humans, the consequences of accidental skin exposure are therefore negligible.
Accidental self-administration: As HVT-ND-IBD cannot infect humans, the consequences of accidental self-administration are therefore negligible.
Contact to or inhalation of dust particles containing HVT-ND-IBD: As HVT-ND-IBD cannot infect humans, the consequences of exposure are therefore negligible.

It can be concluded that the consequence of exposure to the GMO is negligible and therefore the risk is effectively zero.

Please note: as the vaccine is contained in glass ampoules stored in liquid nitrogen and there are reports that ampoules might explode when removed from the cold storage and thawed, this could potentially lead to skin cuts by the glass. So, like for the other cell associated Marek's disease vaccines stored deep frozen in ampoules, an appropriate warning has been included in the product literature to inform the user to protect hands with gloves, wear long sleeves and use a facemask or goggles during ampoule thawing and opening. This hazard is however not directly related to the vaccine, but to the glass ampoule.

Control of risk

Besides the warning for the opening of the glass ampoules which is not related to the vaccine itself, no precautions for use are needed as the risk to humans is effectively zero.

2. Assessment of risk to the environment

In the exceptional case that avian species may become infected with the vaccine strain, no negative effects are expected due to the nonpathogenic nature of the GMO. So the consequences for such an animal are negligible.
As a live HVT vaccine, the vaccine strain is excreted from birds (although at a lower level than the parent HVT strain) which will be vaccinated and may spread to turkeys if they would come into contact with dust particles containing the vaccine. Therefore, precautionary measures will be followed during the field trials in order to avoid direct or indirect contact between vaccinated chickens and turkeys or other avian species.

The overall risk to the environment is effectively zero.

Risicomanagement maatregelen

5.6. Welke criteria worden gehanteerd bij de selectie van proefdieren en wat is het effect van deze criteria op de milieuveiligheid?

No selection criteria related to the protection of the environment will be used for the selection of chickens used in the studies, except that birds must be kept in a closed chicken house according to standard farm management procedures (described in 5.8). Chickens that will be used represent the type of birds commonly used for broiler production.
The type of chickens used has no impact on the health and environmental aspects of the study.

5.7. Beschrijf welke maatregelen voorzien zijn ten aanzien van isolatie van het proefdier.

Housing (~isolation) of the study chickens will be according to standard farm management procedures (described in 5.8). Only the study chickens are allowed in the chicken house. These birds will have no contact with other birds (including turkey and wild birds, also see 5.8 third bullet) and unauthorized visitors are prohibited. At the poultry farms concerned and in the near surroundings no turkeys are kept, which excludes the possibility of spread to these birds through e.g. dust.

Transport of birds (e.g. to the facilities of MSD AH in Boxmeer) will be conform 'bijlage 1, van de Regeling GGO. Birds will be transported in closed boxes with ventilation openings that are supplied with proper filters. The transport unit used will be locked and will be disinfected after transport.

5.8. Beschrijf welke maatregelen worden getroffen om verspreiding van het GGO naar derden (waaronder bij de studie en de proefdieren betrokken personeel) te voorkomen.

The following standard farm management procedures will be applied, which will be sufficient to prevent spreading of the GMO.
This means:

- Training of farm staff.
- Stable closed to unauthorized persons.
- All (air) vents closed with gauze so no birds can enter.
- Registering of all visitors through a log.
- A hygiene sluice.
- Use of disposable coveralls and footwear (study personal and visitors) or change into study house specific clothing (farm staff)
- Potentially contaminated materials used (such as chicken crates) will be disinfected at the farm.
- All vaccinated birds will be euthanized at the end of the study and be submitted for destruction (see 4.9).
- Manure and litter of the study house will be incinerated at a combustion plant

In conclusion, there is no risk of spreading of the GMO to third parties.

Handelingen bij onverwachte situaties en ernstige voorvallen

5.9. Beschrijf welke procedures gevolgd worden indien er om veterinaire redenen wijzigingen in het risicomanagement noodzakelijk zijn.

Changes in risk management due to veterinary causes are not applicable. Individual diseased or sick chickens will, if possible, be treated in the chicken house or will be euthanized if treatment is not possible. Birds will not be taken outside the chicken house for treatment.
Birds that died or needed to be euthanized will be stored frozen on site and will be destroyed by incineration at the end of the study (see section 4.9).
When it is deemed necessary to perform post mortem examination to determine the cause of a particular disease problem, carcasses will be transported in sealed bio-containers to MSD Animal Health and presented for post mortem examination. These activities will take place under permit IG 98-085 and are not part of this application.

5.10. Beschrijf welke nazorg wordt gegeven indien een proefdier de studie voortijdig beëindigt.

This is not applicable for this study: no specific after-care will be given to the birds;. When birds turn sick during the study they may be treated or will be euthanized and taken out of the study. All dead / euthanized animals will be frozen and stored on site and at the end of the study with all the other animals presented for destruction (see section 5.9 and 4.9).
It is expected that a small number of birds will die during the study as the normal mortality rate in broiler flocks in the Netherlands varies between 2.5 and 4.5%.

Monitoring

5.11. Beschrijf hoe de monitoring wordt opgezet om eventuele verspreiding van het GGO of nucleïnezuur preparaat waar te kunnen nemen.

Since the GMO can only spreads to turkeys and no turkeys are kept on the involved farms and near surroundings, monitoring has no added value.

6. Conclusies van mogelijke milieueffecten

1. Waarschijnlijkheid dat het GGO in natuurlijke habitats persistent en invasief wordt onder de omstandigheden van de voorgestelde introductie(s).

The potential of the GMO to become persistent or invasive is very unlikely. The GMO has not acquired properties that are not already present in the parental HVT FC126 virus. The parental HVT virus is not persistent or invasive but it is a nonpathogenic virus.

The risk/likelihood that the GMO will become persistent or invasive is effectively zero.

2. Selectieve voordelen of nadelen die op het GGO worden overgedragen en de waarschijnlijkheid dat zulks geschiedt onder de omstandigheden van de voorgestelde introductie(s).

The potential of the GMO to obtain new traits would only be possible by recombination with other viruses present in the chickens. It has been demonstrated that HVT-ND-IBD is stable when passaged; see 5.2 (b). Besides the expression of the NDV F gene and the IBDV VP2 gene, HVT-ND-IBD has not acquired biological properties that are not already present in the parent HVT FC-126. As HVT does not appear to recombine under natural conditions with other viruses, the possibility that new traits are introduced in the GMO by recombination are effectively zero.

Therefore, the likelihood that the GMO will acquire properties that render it less safe than the parental HVT FC-126 is effectively zero.

3. Kans op genoverdracht op andere soorten onder de omstandigheden van de voorgestelde introductie van het GGO en selectieve voordelen of nadelen die op deze soorten worden overgedragen.

The potential of the GMO to transfer genes to other species would only be possible by recombination with other viruses present in the chickens which will be vaccinated or by integration in the chicken genome. Besides expressing the NDV F gene and the IBDV VP2 gene, HVT-ND-IBD has not acquired biological properties that are not already present in the parent HVT FC-126. Despite decades of intense poultry husbandry and co-infection of commercial poultry with MDV1, MDV2, and HVT, no evidence of interspecific recombination among these avian herpesviruses or other viruses is available. As HVT does not appear to recombine under natural conditions with other viruses, the possibility that new traits are introduced in other viruses by recombination are effectively zero.
To date, there is no evidence that HVT integrates into the genome of chickens or turkeys; HVT infection is not oncogenic, and studies with HVT have never revealed integration into the genome.
Therefore, the likelihood that the GMO will transfer genes to other viruses or to the chicken genome is effectively zero.

4. Mogelijke onmiddellijke en/of vertraagde milieueffecten van de directe en indirecte interacties tussen het GGO en niet-doelwitorganismen.

As outlined above in section 5 (milieurisicobeoordeling) above, the risk for the environment is effectively zero. If under the proposed use the vaccine would enter in the environment and contact with turkey would occur, there will still be no impact as the GMO is fully nonpathogenic for turkeys. Furthermore, it can be noted that the parent virus HVT is endemic and ubiquitous in domestic turkeys and shedding of the HVT-ND-IBD virus is less compared with the parental HVT

strain; therefore, the likelihood of subsequent spread of the vaccine virus in turkeys is effectively zero.

5. Mogelijke onmiddellijke en/of vertraagde effecten op de menselijke gezondheid van mogelijke directe en indirecte interacties tussen het GGO en personen die werken met, in contact komen met of in de nabijheid komen van de GGO-introductie(s).

As outlined above in section 5 (milieurisicobeoordeling) above, the risk for humans is effectively zero. The HVT vaccine strain, HVT-ND-IBD can only infect avian species and does not infect humans. Therefore there is no effect of exposure of persons working with the vaccine or in close proximity to the site of introduction.

6. Mogelijke onmiddellijke en/of vertraagde effecten op de gezondheid van dieren en effecten op de voeder/voedselketen van consumptie van het GGO en alle daarvan afgeleide producten indien deze voor diervoeder bestemd zijn.

As outlined in section 5 (milieurisicobeoordeling) above, there is no risk on the health of avian or other animals. The vaccinated study chickens will not be used for consumption and will be destructed in line with applicable regulations. However, it can be noted that vaccinated chickens would be safe for consumption.

7. Mogelijke onmiddellijke en/of vertraagde effecten op biogeochemische processen die veroorzaakt worden door mogelijke directe en indirecte interacties tussen het GGO en doelwit- en niet-doelwitorganismen in de nabijheid van de GGO-introductie(s).

No biochemical processes which could be induced by the GMO are known.

8. Mogelijke verandering in de staande medische/veterinaire praktijk.

No changes will take place in current veterinary practice.