**Summary of GMO product environmental risk assessment**

**Nobilis® rHVT-ILT (Innovax®-ILT)**

1. **Introduction**

Nobilis® rHVT-ILT is an active recombinant vaccine containing Herpesvirus Turkey (HVT) strain FC-126 which inserted by gD and gI gen from Infectious Laryngotracheitis virus (ILT) strain USDA Challenge Lot No. 83-2. HVT virus induced immunity against Marek’s Disease (MD), gD and gI genes from ILT virus inserted will expressed gD and gI protein which induced immunity against ILT virus. GMO vaccine Nobilis® rHVT-ILT gives double protection against Marek’s and ILT disease.

The advantages of Nobilis® rHVT-ILT application compared to the conventional vaccine is provide protection up to 60 weeks against Marek’s disease and ILT without causing post vaccination lesions nor latent infection. Application of this vaccine do not interfere with other vaccine which given before or after.

Nobilis® rHVT-ILT is manufactured by Intervet Inc. (Merck Animal Health) USA, which registered on other countries as Innovax®-ILT or Innofusion®-ILT or Fowl Larygotracheitis-Marek’s Disease Vaccine Serotype 3, Live Marek’s Disease Vector. Nobilis® rHVT-ILT will be marketed by PT Intervet Indonesia for control and mitigation of Marek’s Disease (MD) and Infectious Laryngotracheitis (ILT) in chickens in Indonesia.

Nobilis® rHVT-ILT has been registered and obtained Free Sales Certificate (FSC) in various countries, such as USA (2007); Peru (2008); Argentina and Bolivia (2009); Canada, Colombia, and Nepal (2010); Brazil, Costa Rica, Kazakhstan, Mexico, Philippines, Russia, and Thailand (2011); Ecuador (2012); Belarus and Ukraine (2013); Bangladesh and South Africa (2014); European Union (2015).

Based on Government Regulation No. 21 Year 2005 concerning Biosafety of Genetically Modified Organisms, and Regulation of the Minister of Environment No. 25 Year 2012 concerning Guideline for Preparation of Environmental Risk Assessment of Genetically Modified Organisms, then the Biosafety Technical Team for Genetically Modified Organisms has conducted Environmental Safety Assessment of Nobilis® rHVT-ILT vaccine. This assessment was based on genetic information and environmental safety information which consists of modified genetic properties, genetic stability of the GMO, potential spread of the GMO, natural host of the parental microorganisms of the GMO, and possibility to pose a risk to the environment, as described in below paragraphs.

1. **Information of Genetically Modified Organisms (GMO)**

**II.1. General description of the GMO**

The GMO used in the construction of Nobilis® rHVT-ILT is Herpesvirus of Turkey (HVT) as the parental microorganism which carries the inserted gD and gI genes of the Infectious Laryngotracheitis Virus (ILT) as the donor gene.

**II.2. Characteristic information of the GMO**

The parental microorganism of the GMO is HVT serotype 3 strain FC-126 (NCBI accession number AF291866) which is a non-pathogen Marek’s disease virus strain and has been widely used as the vaccine strain to prevent Marek’s disease (Calnek *et al.,* 1997).

Gene donor of Nobilis® rHVT-ILT is ILT virus strain USDA Challenge Lot No. 83-2 with the accession number in the gene bank U28832, whereas the inserted gene is gD and gI genes, with gD protein identity number AAC55100.1 and AAC55101.1 for gI protein (Wild *et al.*, 1996). Both of the type-I membrane protein was expressed on the ILTV virion surface, where gD function as the adhesion process mediator and virus penetration to the host cells and plays an important role in ILTV replication, while gI plays a role in virus spreading from cell to another cell (Pavlova *et al.*, 2013). Efficacy study of Nobilis® rHVT-ILT (*Study Report 11. Efficacy testing by in ovo administration - Study One,* 2008; dan *Study Report 12. Efficacy testing by in ovo administration - Study Two,* 2008) shows that these both proteins are the good vaccine candidate.

The flanking region is located in the open reading frame (ORF HVT-064) which is a non-essential region of the HVT. One copy of the ILTV genome fragment which containing glycoprotein D and I was inserted into the HVT genome on the *Xho*I restriction site located in nucleotide 111,241 HVT genome, without alter the gene structure. This insertion doesn’t have an adverse effect and does not affect the parental virus, even provide additional protection against ILT in chicken.

In 5 (five) times back-passage studies in-vitro on chicken embryo fibroblast (CEF) and in-vivo on natural host (chickens), it was proven that gD and gI genes on HVT FC-126 genome was phenotypically stable using RT-PCR method, and genotypically stable using southern hybridization. This shows that there is no possibility of the viruses back to virulent in Nobilis® rHVT-ILT vaccine (*Report 7: Genetic and phenotypic stability of HVT/ILT-138 following back passage through the natural host,* 2008).

**II.3. Methods of the Genetic Construction**

Cloning strategy used in construction process of the GMO HVT/ILT-138 which expressing gD and gI heterologous proteins is homologues recombination methods from overlapping genomic fragments of HVT (*Report 13R/0279. Construction of the HVT Vector Vaccine Innovax-ILT (HVT/ILT-138),* 2014).

Construction process of the GMO are divided into three major steps, as follows:

1. Generation of the DNA cosmid library with overlapping sub-genomic fragments of HVT strain FC-126.

HVT DNA was sheared and size selected on a glycerol gradient as described by van Zijl *et al* (1988) with 40–50 kb fragments chosen as the insert population. Sheared fragment was given blunt ends, then were ligated to a pWE15 cosmid vector, which had been digested with BamHI, and made blunt ends. Selection of fragment using specific probes, labeled using a non-radioactive system, obtained three cosmids which contains the target genome fragments that does not contain the *Not*I restriction enzyme site, such as pWE15 407-32.2C3, 407-32.1C1, and 407-32.5G6. Furthermore, Cosmid-pWE15 407-32.1C1 was used to generate plasmid-pNEB193 672-01.A40, 672-01.C40 flanking the plasmid homologue vector pNEB193 711-92.1A which contains insertion of gD and gI encoding genes of ILT virus on ORF HVT064.

Two fragments of HVT genomes, each has two *Not*I restriction enzyme site, were produced from sheared by *Bam*HI restriction enzyme. Both fragments were further cloned on different cloning vectors. *Bam*HI#1 fragment which sized 29 kbp was initially cloned on cosmid pWE15 in unique site *Bam*HI, then sub-cloned on cosmid-pSY1005 via *Bam*HI restriction enzyme site, generating cosmid-pSY1005 415-09.BA1. Whereas the 26 kbp sized fragment of *Bam*HI#II was ligated to unique site *Bam*HI of plasmid-pSP64, resulting in plasmid-pSP64 172.07.BA2.

1. Generation of homologue vector containing the insertion of gD and gI encoding genes of ILT virus.

The insertion vector of HVT was prepared by ligating of HVT strain FC-126 genome fragment sized 8.6 kbp, resulted from shearing by *Asc*I restriction enzyme in Cosmid-pWE15 407-32.1C1 at *Asc*I site from derivative shuttle plasmid pNEB193 (586-36.6) resulting in plasmid 633-93.2. This plasmid was modified by inserting a *Pac*I short sequence in *Xho*I site (position 111,241 in HVT genome, AF291866) resulting in plasmid 654-45.1. This short sequence consisted of *Pac*I site which was used for the insertion and expression of the foreign genes, where the *Pac*I identification site was flanked by the *Xho*I restriction site.

The 7,971 bp genomic fragment from an *Asp*718I digest from the ILTV *unique short region* (US) which contains gD and gI genes was cloned into the *Asp*718I (*Kpn*I) site of the pUC 19 plasmid: the resulting plasmid 453-48.8A. Digestion using restriction enzyme *Sal*I and *Hind*III into plasmid 453-48.8A resulting a 3563 bp fragment containing gD and gI genes. *Sal*I-*Hind*III fragment was cloned in the same restriction site in derivative plasmid between pSP64 (546-93.A3) resulting in plasmid 684-40.B1.

Final step was a Pacl digestion of plasmid 684-40.B1 and isolation of a 3583 base pair fragment containing the ILT gDgI genes and subsequent cloning of this fragment into the Pacl site of HVT insertion vector 654-45.1 resulting in homology vector 711.92.A1 for HVT/ILT recombinant virus construction.

1. The recombinant virus has been constructed by the transfection of chicken embryo fibroblast (CEF) cells culture with cosmid mixture which containing overlapping genomic fragments of HVT.

The following combination of subgenomic clones and enzymes were used:

(1) 407-32.2C3 digested with *Not*I

(2) 172-07.BA2 digested with *Bam*HI

(3) 407-32.5G6 digested with *Not*I

(4) 672-07.C40 digested with *Not*I

(5) 672-01.A40 digested with *Not*l

(6) 711-92.1A uncut

(7) 415-09.BA1 digested with *Bam*HI.

The cosmid mixture were transfected by lipofection system into the CEF cells. Selection and purification of the recombinant virus originated from the formed plaques were done three times resulting in Nobilis® rHVT-ILT virus vaccine.

**II.4. Characteristic of Genetic Modification**

Insertion of DNA sequences of gD and gI ILT viruses into the HVT strain FC-126 genome does not have an adverse effect or does not affect the virulence of the parental virus (*SID, Starting Not in Pharmacopoeia Material 2.C.2.1.a.Source, 2008*). This is indicated by the results of testing the GMO vaccine Nobilis® rHVT-ILT which was inoculated subcutaneously in one-day-old chickens that showed no significant difference with the parental virus (HVT strain FC-126) both in the pattern of virus spread in tissues (tissue tropism) and on viral replication ability (*Report 13R/ 0201. Dissemination of Innovax-ILT in One Day Old SPF Chicken Vaccinated Via The Subcutaneous Route, 2014*).

The insertion of the gD and gI genes from the ILT virus, in contrast, provides added value for the use of the GMO vaccine Nobilis® rHVT-ILT as a vaccine, which form protection against Infectious Laryngotracheitis disease in addition to Marek's disease (*Vagnozzi et al., 2012*).

**II.5. The possibility of genes inserted in GMO for animal vaccines transferred to other organisms**

Genes inserted in microorganisms for GMO vaccine Nobilis® rHVT-ILT cannot be transferred to other hosts / organisms, because HVT does not integrate with the chicken genome.

Based on BLAST search results (http: /VT/blast.ncbi.nlm.nih.gov/Blast.cgi) homology is not found with the chicken genome and other organisms. Thus the possibility of HVT genome integration or ILTV insertion sequence in the chicken genome through homologous recombination is very small.

**II.6. Conclusions**

Based on the results of the study of genetic information it can be concluded that:

1. Expressions of the interest genes (gD and gI genes from ILT virus) inserted in the HVT genome did not change the virulence properties of HVT and could provide added value in the form of multiple protection against Marek's disease and ILT.
2. The HVT-ILT recombinant virus contains one copy of gD and gI gene that are genotypically and phenotypically stable.
3. gD and gI genes that are inserted in the GMO (HVT-ILT) cannot spread or move to other organisms.
4. **Environmental Safety Information**

**III.1. Spread Ability of Microorganism**

Microorganisms for the GMO vaccine Nobilis® rHVT-ILT have the same properties as the HVT virus whose parental are not pathogenic and are not excreted in chicken excreta so that they do not pose a risk to the environment (*Report 13R / 0201. Chickens vaccinated via the subcutaneous route, 2014*).

**III.2. Information on Host Coverage Range of GMO virus for Vaccine**

Parental microorganism of the Nobilis® rHVT-ILT vaccine is an FC-126 strain HVT virus. The natural host of the HVT virus is turkey (*Calnek et al., 1997*).

**III.3. Molecular Study Information**

Genetic modification of HVT strain FC-126 with insertion of gD and gI genes from ILT virus. The insertion location is in the open reading frame (ORF HVT-064) which is a non-essential part of HVT. The purpose of this gene modification is to make a GMO vaccine containing recombinant HVT virus that expresses gD and gI proteins of the ILT virus, therefore the use of GMO vaccine Nobilis® rHVT-ILT can induce immunity and provide dual protection against Marek’s disease and ILT.

The HVT-ILT recombinant virus contains one copy of the gD and gI genes that are genotypically and phenotypically stable, and there is no possibility of the spread or transfer of gD and gI genes which are inserted into GMO (HVT-ILT) to other organisms so it safe in the environment.

Markers that can be used to identify GMO are sequences of HVT genome fragments that flank the gD and gI genes from ILT virus. The technique for identifyinf this sequence can use the Polymerase Chain Reaction (PCR) technique and DNA sequencing using primers (*Report 13R/0280. Southern blot characterization of Innovax-ILT, 2014*) as follows:

|  |  |  |  |
| --- | --- | --- | --- |
| **No.** | **Primers** | **Primers Sequences** | **Product Length** |
| 1 | Left gD-Fw (1) | 5’-caacacggtcttcaaagcaa-3’ | 894 pb |
| 2 | Left gD –Rev (2) | 5’-cgctgctcttccacactgta-3’ |
| 3 | Right gI –Fw (3) | 5’-atcctctcaaaaggggcagt-3’ | 889 pb |
| 4 | Right gI-Rev (4) | 5’-gatgtcaccctaatgccaca-3’ |
| 5 | gDgI-Fw (5) | 5’-gctttgtacgcaacgctatg-3’ | 2000 pb |
| 6 | gD-gI –Rev (6) | 5’-gatccacgccattttctagc-3’ |

**III.4. Possibility of Negative Impact of GMO Vaccine Microorganisms on The Environment**

The results of the BLAST nucleotide analysis of the gD and gI genes show that the homology of the genome sequences of other organisms is not found, so the possibility of gene transfer to other organisms is not possible.

GMO vaccine Nobilis® rHVT-ILT does not form spores because the parental of this vaccine are included in the group of viruses. The results showed that there was no spread of the virus through contact between vaccinated chickens and unvaccinated chickens (*Report 5. Shed and Spread of Turkey Vector Vaccine Infectious Laryngotracheitis Herpesvirus, HVT/ILT-138 in Chickens, 2008*).

Vaccine viruses can infect their natural hosts, turkeys, but like the nature of their parental this virus is not pathogenic and does not have the risk of spreading to other species or the environment.

**Conclusion**

1. Biosafety Technical Team for Genetically Modified Organisms of Environmental Safety considers that the proposed GMO vaccine Nobilis® rHVT-ILT is safe for the environment.
2. GMO vaccine Nobilis® rHVT-ILT is safe for the environment because it does not risk spreading microorganisms to the environment.
3. Based on the stability study of genotype and phenotype, GMO microorganisms in the GMO vaccine Nobilis® rHVT-ILT were declared stable.
4. If it has been declared safe in the environment, then the product is proven to pose a risk to human and animal health, the applicant must take control and control measures and destroy the GMO vaccine Nobilis® rHVT-ILT in the Indonesian territory.
5. GMO vaccine Nobilis® rHVT-ILT may not be used as a chicken vaccine before obtaining an Environmental Safety Certificate from the Ministry of Environment and Forestry.
6. If new data and information are found that are not in accordance with the environmental safety data obtained to date, then the environmental security status of the GM vaccine Nobilis® rHVT-ILT needs to be reviewed.

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