Technical Opinion nº 2279/2010

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Applicant: CEVA Saúde Animal Ltda.
CQB: 0256/08
CNPJ: 03.224.570/0001-53
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Matter: Requests opinion on biosafety of a genetically modified organism for activities of import, transport, storage and marketing.
Previous summary: 1766/2009, published on 03.09.2009.
Meeting: 130th CTNBio Regular Meeting held on 02.11.2010.
Decision: GRANTED.

Synopsis: CTNBio, following examination of a request for Technical Opinion on biosafety of a biologic risk Class 1 genetically modified organism for the purpose of import, transport, storage and marketing, was favorable to the GRANTING of the request under the terms of this Technical Opinion. Mr. Paulo Roberto Andreoli, Chairman of the Biosafety Internal Commission of the company CEVA SAÚDE ANIMAL LTDA., requested CTNBio a technical opinion on biosafety of a genetically modified organism to be used as an avian vaccine. The request encompasses activities of import, storage and marketing, by the company in Brazil, of a product styled "VECTORMUNE® HVT-NDV – Live frozen vaccine against Marek's Disease and Newcastle Disease". The product shall be imported ready and finished, whereby the phases of production, purification and packaging take place outside Brazil. The company submitted the appropriate documents for the request.
Voting taken at the plenary meeting resulted in eighteen (18) votes for, three (3) abstentions, and zero (0) vote against the request. As determined by Law nº 11105/2005, regulated by Decree nº 5591/2005, CTNBio held that the product complies with applicable rules and legislation aiming at securing biosafety to the environment, agriculture, human and animal health.

1. General Information

The vaccine for which commercial release is requested contains a live genetically modified virus used as vaccine for Marek's Disease (MD), Maleagrid herpesvirus 1 (HVT), which expresses an important antigen in protecting against Newcastle Disease (ND). Newcastle Disease Virus (NDV), a member of the Paramyxoviridae, subfamily Paramixovirinae, genus Rubulavirus, containing a single stranded RNA genome, is the ethiological agent of ND. The disease is highly contagious, takes commercial birds and other avian species, causes respiratory symptoms (cough, sneeze, death-rattle) often accompanied by nervous manifestations and diarrhea and head swelling. Clinical manifestation and mortality vary according to the virus sample pathogenicity. ND virus pathogenicity ranges from very high (velogenic sample) to intermediary (mesogenic sample) and very low (lentogenic sample). On the other hand, MD is a lymphoproliferative infectious disease, characterized by tumors in nerves, skin, spleen, liver, kidney, ovary, testicle, eye and remaining viscera. In acute cases, birds present severe depression, motor incoordination, and uni- or bilateral paralysis of pelvis members. In chronic cases, there is always paralysis of either one or both pelvis members, and wings and neck may be affected. Sciatic and vagus nerves may be

affected by thickening and changing to a gray or yellowish color. The disease is caused by a Gallid herpesvirus, family Herpesvirinae, subfamily Alphaerpesvirinae.

2. Description of the Genetically Modified Organism

Polymerase Chain Reaction (PCR) was used to amplify a 2.9 kb region of HVT genome that was inserted into pUC18. This 2.0 kb fragment contains one incomplete Open Reading Fame – ORF (UL44) and two complete ORFs (UL45 and UL46). Gene F was isolated from double stranded DNA (cDNA) through conversion of a single strand RNA, NDV negative polarity genome, into a double stranded cDNA. Gene F was amplified using PCR primers, producing a 1680 pb fragment. Gene F was then cloned to a pUC18 vector containing Pec promoter, which was synthesized by linking the enhancer region of previous immediate promoter of cytomegalovirus (nucleotide -582 to 307) and the nuclear sequence of β-actine promoter of chicken (nucleotide -1192 to -922) (Tsukamoto et al. 2002. J. Virol. 76:5637-5645). A fragment containing gene F and Pec promoter and a fragment containing the SV40 polyadenylation signal sequence (Griffin, 1981) were inserted to site SfiI generated from the insertion site. Chicken embryo fibroblasts (CEF) were used as host cell for recombining homologue plasmid and genomic DNA. After transfection, the growing virus in CEF was purified by limiting dilution. Individual plaques were expanded in duplicate and selected for expression of gene F. The selection process was repeated until the pure recombinant virus was obtained.

3. Product Biosafety

Analysis of the GMO according to Ruling Resolution nº 5, of March 12, 2008, Annex III

1. The disease to be controlled with the use of the vaccine and the host species, indicating the organs colonized by the vaccine, when live, and the host species of the parental organism from which the vaccine was constructed.

Marek's Disease and Newcastle Disease are the diseases to be controlled. Birds are the host species of the parental organisms originating the vaccine.

2. Immunity level and duration produced in the host species after vaccination with the GMO, informing the time during which the GMO may be detected in vaccinated animals and their excrements, providing experimental evidences.

It was demonstrated that the recombinant vaccine is safe for use in chickens. Embryos up to 18 days were vaccinated in ovo and one 10X dose of the vaccine was administered. After an observation period of twenty-one days, no adverse reactions or clinical signs of MD or ND were recorded.

Safety of the vaccine was tested by inoculating in ovo one 10X dose. After hatching, the birds were observed for twenty-one days for clinical signs of MD and ND. As a control, a group of birds was observed as negative control and another group was inoculated with a challenge sample of very virulent NDV sample, RB1B. After 120 days neither the negative control group nor the group inoculated with the recombinant vaccine displayed signals characteristics of ND. Besides, the weight of the vaccine group birds and control birds did not record any statistically significant difference.

Vaccine safety was also assessed during an efficacy study. Birds were vaccinated with one day of life and maintained for four weeks for immunity developing before challenging. During this period, birds were observed on a daily basis and no adverse reactions or clinical signs of MD or ND were recorded. After the observation period, the birds were challenged with NDV and the vaccine proved efficient.

3. Possible dissemination of the vaccine organism from inoculated to non-inoculated animals or to other species, including humans, informing the mechanisms and frequency of the event with experimental data.

Literature data demonstrated that transmissibility of HVT is limited to chicken to chicken due to the limit of viruses present in bird's feathers follicular epithelium (Cho, 1975, Avian Diseases 19, 136-141; Zygraich and Huygelen, 1972, Avian Diseases 16, 793-798). Contact transmission safety of the recombinant vaccine from inoculated to non-inoculated birds was assessed by:
(1) transmission by contact to non-inoculated birds in the presence of HVT in white blood cells (WBCs);
(2) comparison of recombinant vaccine transmission with parental HVT sample.
Chicken were vaccinated with in ovo 10X dose. Upon birth, non-inoculated birds started their contact with vaccinated birds for three weeks. During the period, neither adverse reactions to the vaccine nor clinical signs of MD or ND were recorded. In different times during the three week period, vaccinated and non-vaccinated birds were bled and the leukocytes fractioned for viral isolation in chicken embryo fibroblast (CEF). Virus from vaccinated birds was isolated at all times, while no virus was isolated from non-vaccinated birds. Similar results were obtained for the parental HVT group. The conclusion was that neither the recombinant vaccine nor the parental HVT was transmissible.
Tissue tropism of the recombinant vaccine was assayed for likelihood that an insertion of NDV gene into the HVT genome could cause HVT tropism changes. Chicken were inoculated with a 100X dose of the recombinant vaccine or equivalent amount of parental HVT strain and viral isolation was conducted in different tissues. Birds inoculated with the recombinant vaccine failed to show both adverse reaction and clinical signs of MD and ND during the twenty-one days after inoculation (dai) and macroscopic lesions of MD or ND. On the tenth and twenty-first dai, the recombinant vaccine was isolated from leukocytes, spleen, thymus and bursa. Similarly, a parental HVT sample was isolated from these same tissues at these times. Based on the study, one concluded that the recombinant vaccine tissue tropism was similar to that of the parental HVT sample.
Safety studies were conducted in non-target animals with a 10X dose of the recombinant vaccine in turkeys, quails, pheasants and pigeons. According to literature, one would expect the HVT to multiply in turkeys, since HVT was originally isolated from this bird (Witter et al., 1970 J. Natl. Cancer Inst. 53, 1731-1742) and is omnipresent in domestic turkeys, though non-oncogenic; ; (Witter et al., 1972 Journal of the National Cancer Institute 49, 1121-1129; Calnek & Witter, 1997 Marek's Disease. In: B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif (Eds), Diseases of Poultry, 10th ed., pp. 369-413. Iowa State University Press, Ames, Iowa.) According to the literature, experimental MD infections showed that quails and pheasants are susceptible to the infection (Calnek & Witter, 1997 Marek's Disease. In: B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif (Eds), Diseases of Poultry, 10th ed., pp. 369-413. Iowa State University Press, Ames, Iowa.) HVT host range is less defined than that of MD for experimental infection of different birds, although better defined in tissue culture. HVT is known to multiply in primary cells of chicken, duck and quail, as well in cells of quail lineage (Cowen and Braune, 1988 Avian Diseases 32, 282-297; Lee, 1971; Samorek-Dziekanowska, 1977 Bulletin of Veterinary Institute Pulawy 21, 10-16).
Safety was demonstrated in other avian species (turkeys, quails, pheasants and pigeons) through:
(1) vaccination with either recombinant vaccine or parental HVT sample; and
(2) comparison of clinical signs, microscopic lesions, adverse reactions, and virus isolation between the two vaccinated groups.

Results demonstrated that other avian species, inoculated with the recombinant vaccine, failed to record clinical signs, macroscopic lesions and adverse reactions. The recombinant vaccine was isolated from leukocytes at all times for five weeks from all avian species. Identical results were obtained when avian species were inoculated with parental HVT sample. Based on such results, it was demonstrated that the range of the recombinant vaccine host is similar to that of the parental HVT sample. Unsuccessful attempts were conducted to multiply HVT in different mammal species such as newborn hamsters, rhesus monkeys and marmosets (Calnek & Witter, 1997 Marek's Disease. In: B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif (Eds), Diseases of Poultry, 10th ed., pp. 369-413. Iowa State University Press, Ames, Iowa; Sharma et al., 1972 J. Natl. Cancer Inst. 49, 1191-1197). In addition, researcher attempts to multiply HVT in primary culture and lineage cells of mammals failed to detect evidence of virus multiplication, even after six weeks and ten blind passages (Meulemans et al., 1973 Journal of Comparative Pathology 83, 605-608; Witter & Sharma, 1974 J. Natl. Cancer Inst. 53, 1731-1742). There are no known records on viral or natural isolation of NDV from mammal species. The occurrence is supported by the fact that the host range for the Birnaoviridae family is limited to avian, fish and insect species.

Safety was demonstrated in mammal lineage cells: murine, canine and porcine. These mammal lineage cells were inoculated with the vaccine, and underwent five passages. No cytopathic effects were recorded, neither in the lineage cells nor in any passage. Similar results were recorded when these species were inoculated with the parental HVT sample. The conclusion, based on these results, is that the vaccine host range was similar for the HVT parental sample.

4. Details, as the case may be, of host susceptibility to the vaccine organism affected by the general conditions (for instance, immunosuppression or concomitance with another disease) or by drug treatment or other treatments.

Not applicable.

5. Experimental evidence that the genetic material of the vaccine organism was fully or partially integrated to the genome of the vaccinated host cells.

Not applicable, since the virus is unable to integrate to the host genome.

6. Likelihood of the viral vaccine to revert to a feral state, through recombination or complementation with other intra-cell viruses, providing experimental results in case the event does occur.

Reversion with gene loss would lead to generation of the MD vaccine currently inoculated in all avian world. However, safety studies associated to vaccine genetic stability and purity were also conducted. Lack of virulence reversion was demonstrated and that the vaccine is genetically and phenotypically stable after five successive retro-passages in chicken. No adverse reactions or clinical signs of MD or ND were recorded during each passage or for forty-five days at the group of the fifth passage. In vitro stability of the vaccine was ratified using molecular tests to verify IBDV gene insertion stability (Southern blot analysis) and genetic expression (Western blot analysis and Black Plaque Assay).Southern blot analysis of DNA isolated from the vaccine of fifth retro-passage group evidenced the presence of NDV F gene insertion and verified that the gene insertion was stable in the HVT genome. In order to verify the in vitro gene insertion stability, the vaccine underwent five in vitro passages. Using the same molecular tests already described to verify gene insertion stability (Southern blot analysis) and gene expression (Immunosuppression analysis and Black Plaque assay), the recombinant vaccine proved to be genetically stable in vitro.

7. Possible adverse effects of the vaccine on pregnant animals and its teratogenic

potential, describing the efficiency and innocuity tests conducted.
Not applicable, since the vaccine is indicated to be applied in ovo.
8. Likely interference of the vaccine organism with efficacy of other or subsequent immunizations against other diseases.
The recombinant shows precisely to be efficient for the two infections, MD and ND.
4. Environmental Safety
The data submitted by applicant on stability, non-reversion to virulence during passages in the target-organism and inability to keep in the environment make this vaccine safe for human and animal health and harmless to the environment.
For the foregoing and given the wide use of HVT as a Marek's Disease attenuated vaccine for over thirty years, coupled with the added advantage of protecting birds against Newcastle Disease, the vaccine may be held safe for birds, consumption of inoculated birds and the environment. Therefore, we reached the conclusion that the activity is neither a potential cause of significant degradation to the environment nor harmful to human and animal health.
6. Bibliography
1. Alexander, D.J. 1997. Newcastle Disease and other avian paramyxoviridae infections. In: B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif (Eds), Diseases of Poultry, 10th ed., pp. 541-569. Iowa State University Press, Ames, Iowa.
2. Calnek, B. W. & Witter, R. L. 1997 Marek's disease. In Disease of poultry, 10th edn (ed. B. W. Calnek), pp. 369-413. Ames, IO: Iowa State University Press.
3. Darteil, R., Bublot, M., Laplace, E., Bouquet, J. F., Audonnet, J. C., Riviere, M. 1995. Herpesvirus of turkey recombinant viruses expressing infectious bursal disease virus (IBDV) VP2 immunogen induced protection against an IBDV virulent challenge in chickens. Virology 211, 481-490.
4. Heckert, R. A., J. Riva, S. Cook, J. McMillen, and R. D. Schwartz. 1996. Onset of protective immunity in chicks after vaccination with a recombinant herpesvirus of turkeys vaccine expressing Newcastle disease virus fusion and hemagglutinin-neuraminidase antigens. Avian Dis. 40:770–777.
5. Morgan, R. W., J. Gelb, Jr., C. S. Schreurs, D. Lutticken, J. K. Rosenberger, and P. J. Sondermeijer. 1992. Protection of chickens from Newcastle and Marek's diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein. Avian Dis. 36:858–870.
6. Morgan, R.W., J. Gelb, Jr., Pope, C.R. and Sonderneijer, P.J.A. (1993) Efficacy in chickens of herpesvirus of turkeys recombinant vaccine containing the fusion gene of Newcastle Disease Virus: Onset of protection and effect of maternal antibodies. Avian Diseases 37, 1032-1040.
7. Reddy, S. K., J. M. Sharma, J. Ahmad, D. N. Reddy, J. K. McMillen, S. M. Cook, M. A. Wild, and R. D. Schwartz. 1996. Protective efficacy of a recombinant herpesvirus of turkeys as an in ovo vaccine against Newcastle and Marek's diseases in specific-pathogen-free chickens. Vaccine 14:469–477.
8. Ross, L.J.N., Binns, M.M., Tyers, P., Pastorek, J., Zelnik, V. and Scott, S. (1993) Construction and properties of a turkey herpesvirus recombinant expressing the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. Journal of General Virology 74, 371-377.
9. Sakaguchi, M., H. Nakamura, K. Sonoda, H. Okamura, K. Yokogawa, K. Matsuo, and K. Hirai. 1998. Protection of chickens with or without maternal antibodies against both Marek's and Newcastle diseases by one-time vaccination with recombinant vaccine of Marek's disease virus type 1. Vaccine 16:472–479.
10. Sonoda, K., M. Sakaguchi, H. Okamura, K. Yokogawa, E. Tokunaga, S. Tokiyoshi,

Y. Kawaguchi, and K. Hirai. 2000. Development of an effective polyvalent vaccine against both Marek's and Newcastle diseases based on recombinant Marek's disease virus type 1 in commercial chickens with maternal antibodies. J. Virol. 74:3217–3226.

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