Technical Opinion no. 2226/2009

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Applicant: CEVA Saúde Animal Ltda.
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: 1754/2009. Published in the Federal Official Gazette nº 39, of 02.27.2009.
Meeting: 129th CTNBio Regular Meeting, held on 12.10.2009.
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 Andreolli, Chairman of the Biosafety Internal Commission of the company CEVA SAÚDE ANIMAL LTDA., requests CTNBio a technical opinion on the biosafety of a genetically modified organism to be used as a avian vaccine. The request encompasses activities of import, storage and marketing, by the company in Brazil, of the product styled "VECTORMUNE® FP-MG+AE – Live lyophilized vaccine against Avian Pox Virus, Mycoplasma gallisepticum and Avian Encephalomyelitis". 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. As determined by Law nº 11105/2005, regulated by Decree nº 5591/2005, the Commission took into account that the experimental protocols and other proposed biosafety measures comply with CTNBio rules and appropriate legislation in effect aiming at securing biosafety of the environment, agriculture, human and animal health.
Consolidated technical opinion of authorization for commercial release of the vaccine "VECTORMUNE® FP-MG+AE, live lyophilized vaccine against Avian Pox Virus, Mycoplasma gallysepticum and Avian Encephalomyelitis".
Technical grounds
The vaccine being analyzed contains a live genetically modified virus targeted to prevent avian pox virus (APV), caused by fowlpox virus (FPV), and Mycoplasma gallysepticum (MG) by inserting the genes of such bacteria in the virus. APV virus was modified by genetic engineering and expresses key antigens that protect MG. The vaccine is presented in a lyophilized form and is recommended for active immunization of healthy chicken for protection against avian pox virus and MG, to be administered by puncturing the bird's wing membrane. A conventional attenuated sample of avian encephalomyelitis (AE) shall be added to this vaccine.
MG infection leads to a chronic respiratory disease in chickens and turkeys and avian pox virus is a disease caused by fowlpox virus. APV affects different birds, including chickens, turkeys, pigeons and others. Live virus efficient commercial vaccines became available on the sixties and smooth strains, safe enough to be used in one-day chicks, were developed in the middle seventies. Although APV is not a respiratory disease, it is

the cause of breathing symptoms and asphyxia. AE is a disease caused by a picornavirus that affects adult and young birds, though only the young ones, up to eight weeks of life, develop the disease characterized by tremors and neck and head paralysis. In egg-producing birds an abrupt decrease in laying is recorded.

Characterization of the Genetically Modified Organism

Receiving microorganism: Fowl Pox Virus (FPV), Cutter strain, considered as the strain of origin of most vaccines against avian poxvirus licensed by the USDA in the United States. APV virus is classified in the Poxviridae virus genus Avipoxvirus. It is an enveloped, double stranded DNA virus, with 300 kilobases (Kb), that causes infection in different avian species.

Donor microorganism: Mycoplasma gallisepticum (MG), strains S6 and R, member of the order Mycoplasmatales, family Mycoplasmataceae and genus Mycoplasma. The gene inserted in the FPV genome codifies a protein that is responsible for stimulating the bird's immune system and the production of antibodies that neutralize the field strains of Mycoplasma gallisepticum. The receiver (FVP) and donor (MG) were considered by the applicant as belonging to group 1.

The cloning site for inserting MG genes, the 40K and mgc3 in the FVP parental sequence is located inside fragment 3.0-kb Hpal-Spel of the FVP parental sample. The 3.0-kb HpaI-SpeII blunt end fragment of the FPV genomic DNA was inserted in a site of the blunt end of EcoRI-HindIII of pUc18. Using EcoRV to digest the 3.0-kb HpaI-SpeII fragment, a fragment of 175-pb was removed and reinserted with MG genes 40k and mgc3 (Yoshica et al., Infect. Immun. 2000 Jun; 68(6):3186-92). Gene 40k was amplified by PCR, jointly with a synthetic promoter Ps and a signal sequence, derived from gene gB of the Marek's Disease Virus (MDV) of serotype 1, was inserted in pUC18 digested by Eco-RV containing the FVP genomic DNA, resulting in an intermediate vector. Promoter Ps emulates the early/late poxvirus promoter (Davidson and Moss, 1989a J. Mol. Biol. 210, 749-769; Davidson and Moss, 1989b J. Mol. Biol. 210, 771-784). The gB signal sequence of MDV was added to the amino terminal region of MG genes 40K and mgc3 to translocate such products to the cell surface. Gene mgc3 was inserted in the intermediary vector, jointly with promoter Ps and the signal sequence gB, resulting in a homologue plasmid. For industrial production of recombinantg FP-MG vaccine, propagation of the virus is carried out in primary chicken embryo fibroblast (CEF) cultures derived from SPF poultry, using the MEM Earle growth medium supplemented by bovine fetal serum (In Diseases of Poultry, 9th ed, pp. 585-586. Edited by B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald & Y. M. Saif. Ames: Yowa States University Press; Outline of Production for Foul Pox Vaccine – Live Virus (Product Code 1621-00) – U. S. Vet. Lic. 368 Bioimune Co. Chicken embryo fibroblasts (CEF) were used as host cell for recombining the homologue plasmid and parental sample of FPV. After transfection, the virus growing in CEF was assessed for expression of MG proteins. Plaques expressing MG proteins were isolated and selected until the pure recombinant virus has been obtained.

CEVA submits the use authorizations for such vaccine granted by the USDA in the United States (06.03.2003), Costa Rica (01.16.2006), Mexico (10.2007) Thailand (12.28.2006), Bangladesh (01.28.2007), Peru (03.14.2007), Colombia (02.12.2008), Ecuador (05.15.2006) and Pakistan (05.09.2005).

Analysis of the GMO microorganism 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.

Fowl pox, the disease caused by Mycoplasma gallisepticum (MG) and avian encephalomyelitis (AE) are the diseases to be controlled. Birds are the host species of the parental organisms from which the vaccine was originated.

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

The vaccine safety for use in poultry was demonstrated. Chicken were immunized by puncturing the wing membrane with a dose 10X of the vaccine. After twenty-one days of observation, there was no record of adverse reactions and clinical signs of PV, MG and AE. Thus, the vaccine is safe for use in chicken and fails to pose any safety risk.

Vaccine safety was also assessed during the efficacy study. Eight week chickens were immunized. The birds were kept for three weeks to develop immunity before the challenge. During this period, they were observed on a daily basis and no adverse reactions of clinical signs of FP, MG or AE were recorded. Expired the observation period, the birds were challenged and the vaccine was shown to be efficient against the challenge with FP, MG and AE. When chickens were vaccinated by wing membrane puncturing with a 100X vaccine dose, neither adverse reactions nor clinical signs of FP, MG and AE were recorded. Besides, no adverse reaction associated to the parental FPV sample was recorded, the same as in the vaccine licensed by the USDA in the United States, which was used to construct the vaccine of interest.

Tissue tropism of the vaccine was assessed to examine the likelihood that a change in the FPV tropism could be caused by inserting the MG gene in the FPV genome. Chicken were inoculated with a vaccine 100X dose or equivalent amount of the parental FPV sample and viral isolations were conducted in different tissues. Birds inoculated with the vaccine failed to develop adverse reactions or clinical signs of FP and MG for ten days post-inoculation (DPI). On the fifth DPI, the virus at the place of inoculation was isolated from chicken inoculated with the vaccine and chicken inoculated with the parental FPV, while no viruses were isolated from the trachea, liver or spleen. At the tenth DPI, no virus was isolated from chicken inoculated with both the vaccine and the parental FPV. Based on these results, a conclusion was reached that the MSV tissue tropism was similar to that of the parental FPV sample. Therefore, the vaccine is safe for use in chicken and fails to pose any safety risk.

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.

Safety of vaccine transmission by contact from inoculated to non-inoculated chicken was assessed in:

(1) transmission by contact when studying FPV efficacy;
(2) transmission by contact when studying MG and AE; and
(3) comparison with transmission to the FPV parental sample.

Chicken were inoculated with a 100X dose of the vaccine. Twenty-four hours post-inoculation, non-vaccinated chicken started their contact with vaccinated chicken for three weeks. During this period, no adverse reaction to the vaccine or clinical signs of FP, MG and AE were recorded. To assess transmission, all birds were challenged with FPV, MG or AE. Vaccinated birds were protected from the challenge while non-vaccinated birds were susceptible. Birds inoculated with the FPV parental sample recorded similar results. The conclusion was that the vaccine and the FPV parental sample was not transmissible.

Safety studies were conducted in non-target animals with a 100X dose of the vaccine in turkeys, quails, game birds and pigeons. According to the literature, FPV is known to

colonize turkeys and chicken and is used to vaccinate turkeys by scarification of the thigh (Tripathy & Reed, 1997 pox. In: Diseases of Poultry, 10th ed. Pp 643-659. Edited by B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald & Y. M. Saif. Ames: Iowa State University Press, Winterfield & Reed, 1985 Poultry Science 64, 2076-2080, Yanagida et al., 1992 Journal of Virology 66, 1402-1408). Also described in the literature is the fact that FPV does not replicate in quails (Winterfield & Reed, 1985). In general, FPV is known as non affecting mammals, even though one case of FPV isolation has been recorded with rhinoceros (Tripathy & Reed, 1997 – Pox. In: Diseases of Poultry, 10th ed. Pp., 643-659. Edited by B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald & Y. M. Saif. Ames: Yowa State University Press.)
Safety was demonstrated in other avian species (turkeys, quails, game birds and pigeons) by:
(1) inoculation with the vaccine or the FPV parental sample; and
(2) comparison of clinical signs, adverse reactions and viral isolation between the two inoculated groups.
The results showed that other avian species inoculated with the vaccine failed to record adverse reactions and no virus was isolated at the place of inoculation, blood and trachea of quails, game birds and pigeons. Identical results also emerged when avian species were inoculated with a FPV parental sample. According to the literature, FPV is known to replicate in turkeys and used for inoculation by thigh scarification (Tripathy & Reed, 1997, Winterfield & Reed, 1985, Poultry Science 64, 65-70, Winterfield et al. 1985, Poultry Science 64, 2076-2080.) In turkeys, recombinant and parental FPV were isolated at the place of inoculation only seven days after inoculation. Based on these results, it was demonstrated that the extension of the vaccine host is similar to the FPV parental sample. Therefore, the vaccine is safe for these avian species and its use in chickens fails to pose security risk to other avian species.
Security 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 in the lineage cells or in any passage. Similar results were recorded when these species were inoculated with the parental FPV sample. The conclusion, based on these results, is that the vaccine host extension was similar for the FPV parental sample. Thus, the vaccine is safe for the mammal species analyzed and its use in chicken poses no safety risk to mammal species.
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 FP vaccine currently inoculated in all avian world. However, safety studies associated to vaccine genetic stability and purity were also conducted. Lack of virulence reversion demonstrated that the vaccine is genetically and phenotypically stable after five successive retro-passages in chicken. No adverse reactions or clinical signs of FP, MG, or AE were recorded during each passage or for twenty-one days at the group of the fifth passage. In vitro stability of the vaccine was ratified using molecular tests to verify gene insertion

stability (Southern blot analysis and DNA sequencing) and genetic expression (Western blot analysis and Black Plaque Assay).
Southern blot analysis of DNA isolated from the vaccine of first retro-passage group evidenced the presence of MG gene insertion and verified that the gene insertion was stable in the FPV genome. In order to assess gene insertion stability in a larger extension, the DNA sequence analysis of different gene insertion areas, such as promoters and the genomic locus of insertion confirmed gene insertion stability.
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 DNA sequencing) and gene expression (Western blot and Black Plaque Assay), the vaccine was 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 outside the productive period.
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 two infections, FP and MG, even when added to a non-recombinant vaccine against Avian Encephalomyelitis.
Opinion:
The application seeks the commercial release of live attenuated avipoxvirus vaccine used to control avian poxvirus in poultry. The disease causes severe losses to bird breeding and this attenuated vaccine is widely used all over the world. The vaccine proposed was constructed by genetic engineering, inserting Mycoplasma gallisepticum in fowlpox virus. Besides, an attenuated vaccine against avian encephalomyelitis will be added.
Data submitted by applicant on vaccine stability, its non-reversion to virulence in passages in the target organism, and its inability to maintain itself in the environment make this vaccine safe for human and animal health.
Considering the history of vaccines containing attenuated avipoxvirus and the wide use of FPV as an attenuated avian pox virus vaccine for over thirty years, coupled with the advantage of protecting birds against Mycoplasma gallisepticum and avian encephalomyelitis, the vaccine may be considered safe for birds, consumption of vaccinated birds and the environment. Therefore, considering that the activity is not a potential cause of significant degradation to the environment nor harmful to human and animal health, CTNBio decided favorably to the request for import, storage, transport and marketing of this live vaccine in a plenary voting where twenty-one votes were favorable and two voters abstained.

Walter Colli
President of CTNBio