Technical Opinion no. 2214/2009

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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.
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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, and its use as avian vaccine, 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., requests CTNBio a technical opinion on the 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 the product styled "VECTORMUNE® FP MG – Live lyophilized vaccine against Fowl Pox and Mycoplasma gallisepticum". 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 submitted by the company comply with CTNBio rules and appropriate legislation in effect aiming at securing biosafety of the environment, agriculture, human and animal health.

1. Identification of GMO

GMO designation: VECTORMUNE® FP-MG – Live lyophilized vaccine against Fowl Pox and Mycoplasma gallisepticum.
Species: Recombinant Fowl Pox Virus (rFP-MG).
Phenotype: Fowl Pox Virus was modified by genetic engineering and expresses key antigens protective of Mycoplasma gallisepticum.
Proposed use: Recommended for active immunization of healthy chicken for protection against Fowl Pox and Mycoplasma gallisepticum, to be administered by puncturing the wing membrane.

2. Protein expressed

Key antigens protective of Mycoplasma gallisepticum.

3. Environment Restriction Area

According to applicant, environment risk is low and no ecological effects are expected from environmental MSV. Exposure of non-target animal species and dissemination of the vaccine into the environment shall be restricted to the use in aviaries.

4. Remarks from the Executive Secretary

Documents submitted include the applicant's original request, compliance with requirements from the Public Health Secretary for Human and Animal Health and adequate documentation of the proceedings under CTNBio Ruling Resolution nº 05.

5. Technical grounds

Fowl Pox is caused by a virus of the Avipoxvirus genus. Poxviruses contain DNA and rank among the larger viruses known. As most of large enveloped viruses, poxviruses are easily destroyed by the majority of ordinary disinfectants.
The Poxviridae family has a virus known as Avipoxvirus that is specific for birds. This genus replicates only at the cytoplasm of bird cells, especially epidermic cells, leading to a disease known as Fowl Pox, characterized by skin blisters filled with fluid. There is only one serotype for Fowl Poxvirus, which makes vaccination against Fowl Pox simple and efficient. Birds are susceptible at any age, and may acquire the infection by direct viral contact. The virus may come from another bird or from mosquitoes (with no replication in this vector). In order to transmit the infection, a crack in the skin is sufficient. The virus develops in the cytoplasm of epithelial cells, causing inflammation and lesions from four to ten days after infection. Mortality is low and dissemination of the disease slow among birds.
Since 1798, when the bovine variola virus was first used to immunize human beings against smallpox, Poxviruses have been extensively studied in order to understand molecular mechanisms, in vitro production of functional proteins and as a tool for vaccination, operating as antigen vectors. Some Poxviruses have their genome (300 kilobases) completely sequenced (as the Virus vaccinia and Virus variola), presenting about 200 genes in a flexible genome, where large amounts of DNA may be removed and replaced by exogenous genes, making room for creating numerous vaccines.
Infection by MG leads to a chronic respiratory disease in chicken and turkeys and Fowl Pox is caused by Fowlpox virus. Fowl Pox is a disease affecting a range of birds, including chicken, turkeys, pigeons and fowls. Efficient live virus commercial vaccines became efficient during the sixties and mild strains, safe enough to be used in one-day chicks, were developed in the mid-seventies. Although Fowl Pox is not a respiratory disease, it causes respiratory symptoms and asphyxia.
Applicant requests the release of a vaccine with Avipox virus, FP strain, used as a commercial vaccine, multiplied in chicken embryo fibroblasts. The FP strain virus was used as a receptor of "MG antigen" gene, originated in Mycoplasma gallisepticum. Avipox virus, FP strain, was attenuated through successive passages in culture.
The cloning site for insertion of gene MG 40K and mcg3 in the FPV parental chain is located within fragment 3.0-kb Hpal-Spel of FPV parental strain. The uncut fragment end 3.0-kb Hpal-Spel of FPV genomic DNA was inserted in the uncut end site EcoR1-HindIII of pUc18. Using EcoRV to digest the fragment 3.0-kb Hpal-Spel, a 175-pb fragment was removed and reinserted with genes MG 40K and mcg3. Promptly, one 40K gene amplified by PCR, coupled with a synthetic Ps promoter and one signal sequence derived from gene gB of the Marek Disease Virus (MDV), serotype 1 GA, was inserted in pUC18 digested by Eco-RV containing the genomic FPV DNA, resulting an intermediate vector. Promoter Ps emulates the consensus early/late promoter of poxvirus. The MDV gB signal sequence was added to the amine terminal of genes MG 40K and mcg3 for translocation of such genetic products to the cell surface. Gene mgc3 was inserted in the intermediary vector together with promoter Ps and signal sequence gB, resulting in a homologue plasmid.
Chicken embryo fibroblasts (CEF) were used as host cell in recombining the homologue plasmid and the parental FPV strain. After transfection, the growing virus in CEF was assayed for expression of proteins MG. Plaques expressing proteins MG were isolated and selected until the pure recombined virus was obtained.
Gene MG was introduced in the viral vector by homologue recombination in the cloning site, interrupting a possible open reading frame (ORF). The cloning site is held as non-essential for viral replication and has no known function in the Avipox strain FP

phenotype. According to applicant, the donor gene and its products fail to present any known pathogenic or toxic properties, as well as known attributes that may transmit properties resistant to the receiving virus to any known therapeutic agent.
Equally, poxvirus synthetic promoters fail to have pathogenic or toxic properties. FP-MG Avipox virus is genetically stable in vivo, assessed after five retro-passages in chickens. Tests indicate that the FP-MG Avipox virus is not permissible in mammal cells. The use of doses 10x larger than recommended failed to induce adverse reactions or clinical signs of Fowl Pox or Mycoplasma gallisepticum in birds vaccinated at the eighth week of life. No virus was isolated in fowls, such as birds, pigeons, quails and turkeys after twenty days of immunization with VECTORMUNE FP-MG, indicating the safety of this vaccine. Therefore, the vaccine is safe for chicken and fails to present any safety risk.
Analysis of the GMO organism under 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.
The diseases to be controlled are Fowl Pox and the disease caused by Mycoplasma gallisepticum (MG). The host species of parental organism from which the vaccine originated is birds.
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 chicken was demonstrated. Chicken were immunized by puncturing the wing membrane with a 10X dose of the vaccine. After twenty-one days of observation, there was no record of adverse reactions and clinical signs of FP, and Mycoplasma gallisepticum MG. 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 or were recorded. Expired the observation period, the birds were challenged and the vaccine was shown to be efficient against the challenge with FP and MG. Thus, the vaccine is efficient for use in chicken and fails to present any safety risk. When chickens were vaccinated by wing membrane puncturing with a 100X vaccine dose, neither adverse reactions nor clinical signs of FP and MG were recorded. Thus, the vaccine is efficient for use in chicken and fails to present any safety risk. Besides, no adverse reaction associated to the parental FPV strain 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 dose 100X 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 strain. 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 assayed in:
(1) transmission by contact when studying FPV efficacy;
(2) transmission by contact when studying MG; 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 and MG were recorded. To assess transmission, all birds were challenged with FPV and MG. 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 were not transmissible. Therefore, the vaccine is safe for use in chicken and fails to present any safety risk.
Safety studies were conducted in non-target animals with a 100X dose of the vaccine in turkeys, quails, fowls 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, fowls and pigeons) by:
(1) inoculation with the vaccine or the FPV parental strain; 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, fowls and pigeons. Identical results also emerged when avian species were inoculated with a FPV parental strain. 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 yet not later. 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 strain. The conclusion, based on these results, is that the vaccine host extension was similar for

the FPV parental strain. 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 of the avian world.
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 and MG 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.
Safety was demonstrated in mammal cell lineages: murine, canine and porcine. The mammal lineage cells were inoculated with the vaccine and underwent five passages. No cytopathic effects were observed in any of the lineage cells or in any passage. Similar results were obtained when these species were inoculated with the parental FPV sample. Based on such results, the conclusion was that the extent of the vaccine host was similar for the FPV parental sample.
Applicant has authorizations to market this vaccine granted by the USDA of 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).
6. 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 the avipoxvirus.
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 poxvirus vaccine for over thirty years, coupled with the advantage of protecting birds against Mycoplasma gallisepticum, 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-two votes were favorable and one voter abstained.

Walter Colli
President of CTNBio