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This is to certify that I, Marco Antônio Rochadel, Official Public Translator, designated and installed in Office according to The Official Gazette of June 23, 1982, page 5428, have received and translated, to the best of my knowledge and belief, a document with the following contents:

Ministério da Ciência e Tecnologia

GOVERNO FEDERAL BRASIL PAÍS RICO É PAÍS SEM POBREZA

Ministry of Science, Technology and Innovation– MCTI

comissão técnica nacional CTNBio de biossegurança

National Biosafety Technical Commission – CTNBio

General Coordination

Technical Opinion no. 4594/2015

Proceedings:	01200.002226/2014-65
Applicant:	Boheringer Ingelheim Química e Farmacêutica Ltda.
CQB:	251/08
Proton:	20581/2014
Address:	Avenida das Nações Unidas, 14171, 18º andar, Torre B, Vila Gertrudes, 04794-000 São Paulo, SP
Matter:	Request for opinion related to commercial release of vaccine composed by Biological Risk Class I genetically modified microorganism
Previous Extract:	4130/2014, of 09.24.2010, published in the Federal Official Gazette

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no. 13, of June 13, 2014.

Meeting: 183rd CTNBio Regular Meeting held on June 10, 2015

Decision: **GRANTED**.

CTNBio, following examination of request for a technical opinion on commercial release of a vaccine composed by genetically modified Class I Biological Risk organism, concluded for its granting on the terms of this Technical Opinion.

Regarding the competences mentioned in Law 11105/05 and its Decree no. 5591/05, CTNBio reached a conclusion that the request complies with CTNBio rules and applicable legislation securing safety of the environment, agriculture, and human and animal health.

Ms. Patrícia Schwartz, technician in charge representing Boheringer Ingelheim Química e Farmacêntica Ltda., holder of Biosafety Quality Certificate (CQB) no. 251/08, requests a commercial release registration of a vaccine composed by a Class I biological risk genetically modified organism "**Bovela® Modified Live Vaccine against Type 1 and Type 2 Bovine Diarrhea Virus"**. The process describes the biosafety conditions for manipulation of this microorganism as well as a formal statement by the technician in charge certifying the truthfulness of information delivered to CTNBio. Applicant Boheringer Ingelheim do Brasil Química e Farmacêutica Ltda., based on the contents of CTNBio Ruling Instruction no. 5, request analysis and issuance of a technical opinion for commercial release of the Modified Live Vaccine against Type 1 and Type 2 Virus Bovine Diarrhea; Filed on 05.05.2014. On April 28,

2014, the company delivered the technical report of the Bovela® product for commercial release.

GMO Description

Bovela is a vaccine targeted to actively immunize bovines against the disease caused by the bovine viral diarrhea virus (BVDV) types 1 and 2, includes vaccination of cows and calves, since it also protects the fetus against transplacental BVDV infection. Parental strains of the two viral types were isolated from animals infected in Germany (KE9, BVDV-1) and the United States (NY-93 and BVDV-2). This virus is a single strand positive-sense RNA pestivirus that replicates only in the cytoplasm (family *Flaviviridae*). Proteins E^{ms} and N^{pro}, whenever excluded or manipulated in the viral genome, cause attenuation of the respective mutants and growth retardation when compared with the wild-type. The proteins are able to inhibit the interferon dependent (IFN-gamma) immune response type Th1, therefore inhibiting the host immune response. On the other hand, the absence of coding sequences for these viral genome proteins impairs the ability to inhibit the production of interferon, therefore promoting the host immune system. The vaccine is adjuvant-free and targeted for cattle active immunization of pregnant cows and of animals after three months of age, in order to reduce the clinical symptoms of the disease, viremia and viral excretion.

Purpose (objective)

Applicant requests a technical opinion on biosafety of the Bovela® vaccine for commercial registration to actively immunization against BVDV virus against bovine diarrhea.

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GMO Description

The product related to this commercial release is a veterinarian vaccine targeted to actively immunize bovines against the disease caused by the virus of bovine viral diarrhea (BVDV) and aimed at active immunization of caws and calves, in order to protect the fetus against BVDV transplacental infection. Parental strains of the virus, BVDV-1 KE9 and BVDV-2 NY93 were respectively used in the production of the vaccine. BVDV virus is a single strand positive-sense RNA pestivirus belonging to the family *Flaviviridae* that only replicates in the cytoplasm. Pestiviruses are single strand positive-sense RNA viruses with genomes of about 12.3 kb that contain a long open reading frame (ORF) for a polyprotein of about 2000 amino acids that is processed by co- and post-translation in at least 12 mature proteins. BVDV is restricted to biungulated animals (*Artiodactyla* order), such as the domestic species of *Bos Taurus* (bovines), *capra hircus* (caprines). *Ovis aries* (ovines) and *Sus scrofa* (swines), as well as in some species of wild ruminants, including the *Cervus elaphus* (red deer), *Ozotoveros bezoarticus* (Pampas deer), *Odocoileus virginianus* (white-tailed deer), *Bison bison* (bison), *Vicugna pacos* (alpaca) and *Lama glama* (llama), among others.

The genetic transformation suffered by parental viruses was achieved by functional deletion of two genes, to wit: (1) ribonuclease (E^{rns}) gene 349 amino acid coding region deletion, and (2) protein N^{pro} coding gene region deletion, except for the 4 codons of the amino-terminal tail. E^{rns} viron resident viral glycoprotein is excreted by virus infected cells. Exclusion of the only E^{rns} amino acid interferes in its RNase activity. However, N^{pro} is a protein expressed in the

infected cell and leads of degradation of IRF3, an essential fact in the interferon signaling cascade of the host. The genetic changes in BVDVs enable formation of autonomous replicons in the host cells, while disabling the ability to produce infectious virus particles. Besides, both sylvan proteins have the ability to interfere with an interferon response (IFN) type1 in cells infected by different viruses or treated with dsRNA. Therefore, the deletions described above disable the ability, caused by the virus, of repressing interferon production by the host, which leads to a better immune active response by animals. Particularly, whenever the vaccine is used in pregnant females, one expects the fetus transplacental infection by BVDV-1 and BVDV-2 to be prevented and, as a consequence, active immunization of calf since their third month of life, reducing clinic symptoms, viral excretion and minimizing the BVDV-caused reduction in leukocytes count.

Biotechnology used and stability of GMOs

Briefly, modified BVDV-1 and BVDV-s RNA were transcribed *in vitro* from complete clones of cDNA pXYKE-B-NdN (corresponding to the modified BVDV-2) and pKANE99C (corresponding to the modified BVDV-1). Later on, the BVDV strains were propagated in MDBK-B2 cells lineage and the viral RNA was extracted to establish the cDNA standard library and to its extension by polymerase (RT-PCR) chain reaction. The target living virus, modified and attenuated, were obtained by cDNA elaboration after *in vitro* transcription and RNA transfection of MDBK cells. Sequencing of master virus seeds (MVS) to modified BVDVs were used to inoculation in MDBK-B2 cells and, after 48 hours of incubation, the supernatant material was removed and cells

were cleansed and lysed. Total RNA was extracted from cells, examined for integrity and later converted into cDNA for sequencing. Results of sequencing the established MVS confirmed the presence of introduced deletions in N^{pro} and E^{rns}. When compared, the MVS sequences and original isolated KE9 and NY93 showed the presence of just minor differences in nucleotides that were determined to have no influence over the attenuated phenotype.

PCR data obtained for MVS and WVS (MVS + 5) of strains in vaccines of modified BVDV have also confirmed the presence of the introduced N^{pro} deletion. The *in vitro* data were also supported by PCR data obtained in BVDV (virus isolation method) positive samples which showed that the introduced N^{pro} deletion remained stable for several *in vitro* passages. Jointly, the data show that BVDV genomes containing the inserted deletions are genetically stable *in vitro* and *in vivo*.

1. GMO identification

GMO Designation:

The scientific names of strains with the genomic deletions coding for the proteins mentioned are: Bovine Viral Diarrhea Virus 1 (BVDV-1) strain KE9 and Bovine Viral Diarrhea Virus 2 (BVDV-2) strain NY93.

Species

RNA virus, BVDV-01 and BVDV-02 are species of genus *Pestivirus*, family *Flaviviridae*.

Introduction Method and Inserted Characteristics:

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Pestiviruses are single strand positive-sense RNA viruses possessing one genome of about 12,3 Kb, expressing a polyprotein of around 400 KDa, which is co- and post-translationally processed to generate viral proteins. BVDV-1 (strain KE9) was used to produce a clone of infectious cDNA used in attenuated deletions. The same was done for BVDV-2 virus (strain NY-93). The two vaccinal strains related to BVDV -1 and BVDV-2 viruses contain two identical genomic exclusions, the first to exclude most part of gene N^{pro}, which prevents expression of N^{pro} protease at the N-terminal portion and the second, to exclude a codon of gene E^{rns}, which resulted in elimination of the viral ribonuclease. The modified RNA of the two viruses was transcribed *in vitro* from complete clones of the two individual cDNAs (pXIKE-B-NdN - BVDV-2 and pKANE99C - BVDV-1). The modified and attenuated BVDV strains were propagated in MDBK-B2 cell lineages (bovine kidney cell lineage), through RNA transfections. N^{pro} is a protease expressed in the infected cell that leads to degradation of IRF3, an important factor in the signaling pathway for production of IFN-gamma in the host. Exclusion of protein E^{rns} activity results in replicons that are able to replicate RNAs in an autonomous way, though unable to produce infectious viral particles. Experimental data presented by applicant show that genomes containing the inserted deletions are genetically stable *in vitro*, since after several passages in host cells molecular biology experiments were conducted (RT-PCR and nucleotide sequencing) that showed that the extant nucleotide changes remained within the expected range for the positive strand RNA virus and that the changes failed to increase the virus multiplication ability. This is an extremely important datum both regarding development of a stable live vaccine and for the product biosafety issues.

II – Aspects Related to Human and Animal Health

The GMO used in the Bovela® vaccine belongs to Risk Class 1 category (according to Ruling Resolution no. 2, of November, 2006), with low individual level and low collective risk, since it is harmless to human and animal health and fails to cause adverse effects to plants and the environment.

Risk for Human Beings

It is known that BVDV is not infectious to human beings, has no zoonosis potential and has no influence in human health according to OIE – World Organization for Animal Health, 2008. Toxic and allergic effects have not been reported in the literature. BVDV has no ability to infect human cells even when such cells are induced to express receptors that are BVDV, such as bovine CD406. This strengthens the firm barrier that prevents BVDV to infect human beings. Vaccines strains are unable to infect the human being, and thus are not pathogenic to humans, independently of age, health condition or immune status of the individual.

Excipients that are part of the vaccine composition are not pharmacologically active and fail to have any toxic effect after human being exposure. All excipients are described in the pharmacopeia (Farm Eur), and are commonly used in medications for human consumption. Salts (WPBS and physiologic saline) and sugar (sucrose) have no biologic relevance and are metabolized by the organism.

A vaccinated animal that has its general condition affected by immunosuppression or

concomitance with other disease may not have a normal immune response to the vaccine. The deletions within the genome did not increase susceptibility to immunosuppression.

The vaccine organism does not integrate its genome to the host genome DNA. Viruses BVDV-1 and BVDV-2 belong to the family *Flaviviridae* and are formed by one single-stranded positive sense RNA. Replication of the viral genome occurs in the cytoplasm of the infected cells. The life-cycle does not include DNA intermediation and the viruses do not express the reverse transcription. Therefore, DNA recombination of the host cell is not expected.

The BVD type 1 master seed, P05022006 BVD, and BVDV type 2 master seed, ddBVD Tub 2 MSV, batch P14022806, 15/03/06 failed to revert to virulence and are safe to be used in the production of modified live vaccines according to the results observed in studies “nº 6131-0955-06B-90 – Virulence reversion safety study for bovine viral diarrhea type 1 virus double deletion master seed” and “nº 6131-0955-06B-034 - Virulence reversion safety study for bovine viral diarrhea type 1 virus double deletion master seed”. Briefly, both studies pursued to show that there was no reversion to virulence of the BVD type 1 of type 2 master seed with double deletion. A reverted passage model using newborn calves that have not received colostrum and were free from viruses and maternal antibodies passed via the colostrum. The study findings showed that there was not reversion to virulence after 2 and 3 reverted passages for BVDV-2 and BVDV-1, respectively, with any indication of active infection by BVDV.

Studies conducted in pregnant animals showed that vaccination of milk cattle with a BVDV vaccine made from one strain of BVDV-1 and another with a BVDV-2 strain is safe in pregnant

and non-pregnant cows. The vaccination has no effect to conception rates. Regarding efficacy, due to different findings, there is indication of protection in the group vaccinated against field infection by BVDV.

As far as the persistent BVDV infection, it was not established in any of the fetuses and failed to be recorded any influence on the normal development and/or newborns that could be ascribed to this vaccination. There was no record of systemic reaction or grave site after treatment with the vaccine. Based on the results of one of the studies, we can conclude that administration of an overdose of ten times the vaccine with BVDV-1 and BVDV-2 in pregnant female calves in the two semesters of pregnancy is safe.

The Bovela® vaccine is not expected to interfere in the efficacy of other vaccinations, though the use of this product with other vaccines is not recommended.

III – Risk Assessment – Environmental Aspects and Risks to Human and Animal Health

BVDV has low survival ability to survive outside of its host, since it requires a living cell to replicate. BVDV was totally inactivated within a period of 3 weeks at 5^{o}C, 3 days at 20^{o}C and 5 minutes at 50^{o}C in bovine manure and in a pH lower than 5.7 and higher than 9.3. Besides, solar radiation can inactivate members of the *Flavivirus* family in a matter of days. The cycle of this virus does not have a DNA phase (fails to possess reverse transcriptase coded in its genome, since it is not a retrovirus) and the host cells also fail to produce RT. RNA is the only genetic material, and this does not make recombination with the host DNA possible. Therefore, the risk that the viral genome be genetically transferred to the genome of vaccinated animals

or to any other animal or environmental or nosocomial bacteria is very low.

Clinical studies in bovines with each strain of the vaccine were conducted, showing that: 1) immunization started after 3 weeks with 10^4 $TCID_{50}$ of the vaccine in 3 years old cattle; 2) immunity lasted for 12 months for both vaccines; 3) the time of OGMs detection in vaccinated animals showed that the viruses were present in lymphoid organs up to the 13^{th} day after vaccination and in bovine respiratory and gastro-intestinal tissues up to the 6^{th} to the 9^{th} day after vaccination; 4) viremia was recorded between days 6 to 20, and for no longer than the 14^{th} day after vaccination; 5) the modified virus was found in milk traces of vaccinated cows (between days 6 to 23 DAV), though there is not transmission of the virus by milk, since there was no positive serologic response in calves fed with the milk containing the vaccine virus; 6) infections in human beings is not possible since human cells do not possess CD-46, the viral bovine BVDV receptor; 7) there was no reversion to virulence in BVDV virus types I and II, being therefore their use safe in the production of modified viral vaccines; 8) the vaccines are safe to pregnant cows in all phases of the pregnancy and there was not recorded any impact in conception rate, as well as any influence on fetal development (no teratogenic effects); 9) Although the report mentions in its reference list two studies (numbered 2209054 and 2009055) on assessment on duration of fetal protection, the report fails to clarify whether in fact Bovela® provides transplacental protection to the fetus; this point could have been explained in greater detail; 10) live vaccine type I and type II strains provide safety and efficacy to milk cattle vaccination in all stages of pregnancy, in not pregnant cattle and also in

programmed insemination animals; 11) there was no detection of virus in the nasal secretion (which could result in horizontal transmission to the environment), blood, urine, feces in every moment of the studies.

BVDV is an enveloped virus and fails to present resistant structures that may survive, having low survival abilities outside its host. The virus integrity and stability is strongly affected by sunlight (UV radiation), detergents, temperature and pH. Both Bovela® vaccine sylvan and modified BVDV may be inactivated by bleach, alcohol and detergents.

Mutations and deletions that are present in attenuated strains of BVDV-1 and BVDV-2 vaccines help the immune system by developing an adequate response to eliminate the virus. There are no scientific evidences of increased ability of transmission of attenuated BVDV virus for non-target species.

Monitoring

Regarding the post-commercial releasing, CTNBio determines compliance with instructions and conduction of technical monitoring actions mentioned by CTNBio Ruling Resolution no. 09, of December 02, 2011.

IV – Conclusion

Data from scientific literature and results of experiments conducted and submitted by the applying company show that the vaccine Bovela® fails to bring any significant risk to the environment, that it is safe for human and animal health, taking into consideration that:

1- Modified viruses BVDV-1 and BVDV-2 (master seed) fail to present reversion to virulence and the attenuated phenotype is stable;

2- BVDV is no infectious to human beings, does not affect human health and has no zoonosis potential;

3- Vaccine strains were generated by deletions in two genes of the viral genome, N^{pro} and E^{rns}, with no additional external sequence, which means that the transfer of sequences that do not belong to BVDV would be impossible to occur;

4 - Besides, once the BVDV genome is contains just RNA, and has no codification for reverse transcriptase, the potential of genetic transfer to other species' genome is null;

5- Despite the necessary challenge to immunologic protection, due to the heterogeneity existing between the strains of BVDV, both vaccines (live-modified or dead) has proven efficacy in controlling the disease. The vaccination is not 100% effective in each animal, while being effective at the herd level. Although dead vaccines are potentially safer than live vaccines, the latter are less effective and require frequent boosters (at least twice each year).

6 - BVDV is an enveloped virus and fails to have resistant structures that may survive, showing low ability of survival outside its host. Virus integrity and stability is strongly affected by sunlight (UV radiation) detergents, temperature and pH. Both sylvan and modified viruses of the Bovela® vaccine may be inactivated by bleach, alcohol and detergents, minimizing/eliminating the likelihood of causing environmental harm.

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CTNBio finds that the information sent by applicant comply with the provisions of CTNBio Ruling Resolution no. 05 and has the opinion that the product is safe for use and is favorable to the release of the Bovela® vaccine for commercial use. CTNBio understands that the activity is not a cause of significant degradation of the environment and fails to aggravate human and animal health.

Dr. Edivaldo Domingues Velini

CTNBio President

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4- Fairbanks, K. Schnackel, J. Chase, C. Evaluation of a Modified Live Virus Type-1a Bovine Viral Diarrhea Virus Vaccine (Singer Strain) against a Type-2 (Strain 890) Challenge. *Veterinary Therapeutics* • Vol. 4, No. 1, 2003

5- DuBois WR, Cooper VL, Duffy JC, et al: A Preliminary Evaluation of the Effect of Vaccination with Modified Live Bovine Viral Diarrhea Virus (BVDV) on Detection of BVDV Antigen in Skin Biopsies Using Immunohistochemical Methods. *Bov Pract* 34(2):98-100, 2000

6- Novartis – Bovidec – vacina contendo apenas o vírus BVDV-1, inativado com adjuvante e preservado em tiomersal 0,013% (não protege contra BVDV-2).

7- Study no. 2009054: Evaluation of the duration of fetal protection and minimum immunizing dose of a Type-1 and Type-2 BVDV vaccine candidate against virulent Type-1 BVDV challenge 12 month after vaccination, 02/11/2010.

8- Study no. 2009055: Evaluation of the duration of fetal protection and minimum immunizing dose of a Type-1 and Type-2 BVDV vaccine candidate against virulent Type-2 BVDV challenge 12 month after vaccination, 04/07/2011.

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In Witness Whereof, I have hereunto set my hand and seal in this City of Brasília, Federal District, Brazil, this Thursday, March 03, 2016.

Fees according to
Official Gazette of 04/15/2011
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Marco Antônio Rochadel
Public Translator