Objection by African Centre for Biosafety to:

MERIAL SA's Application to the General Release of VAXXITEK®HVT + IBD intended for the vaccination of chickens to prevent Marek's disease and Gumboro disease.

November 2007

Objection by the African Centre for Biosafety in respect of application for VAXXITEK HVT + IBD vaccine (vHVT013-69) for chickens

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The application is for a genetically modified virus (GMO) to be used as a vaccine for chickens. Vaxxitek HVT + IBD is a live vaccine based on the turkey Herpesvirus (HVT) expressing the VP2 transgene from the Infectious Bursal Disease Virus (IBDV). The vaccine is indicated for the active immunisation of chickens to help prevent both Infectious Bursal disease and Marek's disease.

Infectious Bursal Disease is a highly contagious disease of young chickens caused by infectious bursal disease virus (IBDV), and characterized by immuno-suppression and mortality generally at 3 to 6 weeks of age. In recent years very virulent strains of IBDV, causing severe mortality in chickens, have emerged in Europe, Latin America, South-East Asia, Africa and the Middle East http://cost839.var.fgov.be/vir.htm. IBDV is shed in the faeces and transferred by dust and bedding or litter. IBDV is a double stranded RNA virus and there are two distinct serotypes, but only serotype 1 viruses causes disease in poultry. At least six antigenic variants of IBDV serotype 1 have been identified. Many of these variants were reported to evade high levels of maternal antibodies in commercial flocks, resulting in 60 to 100 % mortality rates http://en.wikipedia.org/wiki/Infectious_Bursal_Disease. The IBDV VP2 protein has been shown to contain neutralizing antigenic sites and to elicit a protective immune response in chickens (and hence is was chosen as a vaccine for Infectious Bursal Disease by the applicant). Importantly, most of the amino acid changes between antigenically different IBDVs are clustered in the hypervariable region of VP2; indicating that the virus is constantly mutating or changing this region in order to evade the immune system (Cao et al. 1998; Kasanga et al. 2006). Therefore, the vHVT013-69 vaccine that uses a single IBVD VP2 gene is unlikely to be effective for all IBDV viruses and the virus will evolve or recombine with other IBVD variants to avoid the antibodies generated by the immune system as a result of vHVT013-69 immunisation. In other words, the efficacy of a single recombinant VP2 over time in field conditions will be short lived and a better option would be to use attenuated composite IBDV vaccine or several VP2 recombinant variants.

Marek's disease is a virus-induced disease of chickens characterized by tumor formations in nerve, organ, muscle and epithelial tissue. It usually affects chickens 2 to 16 weeks of age. Clinical signs are "gray eye" caused by tumors in the pupils and blindness, tumors of the liver, kidneys spleen, gonads, pancreas, lungs, muscles and skin. It is a highly contagious and can be transmitted by direct and indirect contact between birds. Transmission is primarily by airborne route as the virus is shed in epithelial cells of the feather follicle, dander, chicken house dust, feces and saliva. The virus has a long survival time in dander since viable virus has been isolated from houses

that have been depopulated for many months. Transmission by egg has no significance (i.e., chicken hatched and reared in isolation will be free of MDV) http://www.addl.purdue.edu/newsletters/2005/Spring/mareks.htm

Marek's disease is caused by Marek's disease virus (MDV) serotype 1, MDV1 (Churchill and Biggs, 1967 and Nazerian et al., 1968). MDVs are typical herpes viruses belonging to the family Herpesviridae (Van Regenmortel et al., 1999) and consist of three closely related viruses serotypes- MDV1, MDV2 and MDV3. The serotype 3 (MDV3) is also known as herpes virus of turkeys (HVT). Marek's disease has been controlled by vaccination since 1970 and is usually very effective (a 90% success rate is reported) http://www.addl.purdue.edu/newsletters/2005/Spring/mareks.htm. Several types of vaccine have been used: attenuated MDV1 (attMDV1), naturally apathogenic MDV2, and HVT (Witter, 2001). The mechanism by which vaccination prevents clinical Marek's disease is poorly understood. Following vaccination of chickens with attenuated MDV1 or HVT, superinfection with field strains of MDV1 has been reported (Churchill et al., 1969; Biggs et al., 1970 and Purchase and Okazaki, 1971). In addition, the co-existence of more than one serotype of MDV in the same host is also an established phenomenon (Cho, 1977). The presence of MDV1 in successfully vaccinated chickens is indicative of infection rather than disease as the host does not develop tumors, but does continue to shed the virus. Therefore, even vaccinated chickens can carry active non-pathogenic viral loads (i.e. carriers of the disease) and the switch from benign to disease-causing virus is not established or understood.

Unintended genetic effects: Uncertain Insertion site and effect on the HBV Genomic integrity and stability

There is a fundamental uncertainty on the host HVT genome as a result of inserting the transgenic cassette containing the IBDV VP2 gene. The site of insertion is described as "intergene1"- presumably an intergenic region between two genes whose functions are unknown. The uncertainty of insertion of the transgene is of increased concern for viruses since overlapping genes are not uncommon and so the consequences are less well understood. Important changes in genome function as a result of gene disruption or mutation of the HVT genome include changes in pathogenicity and host-range. From available scientific evidence and knowledge of viral evolution these changes will occur if there is positive selection. It has been shown that small mutations and differences in intergenic or repeat regions of the MDV1 (compared to non-pathohenic HVT) are probably responsible for pathogenicity (Santin et al. 2006 and Kingham et al 2001).

Therefore comparative genome analysis is required to ascertain if there are any unintended genetic effects/mutations. Experiments using comparative genomics are required to fully establish genome stability of the GM virus, vHVT013-69. Techniques such as repPCR, RAPD and comparative genome hybridization (CGH) will be effective in establishing genome similarity (Bao *et*

al. 1993, Pinkel and Albertson 2005). Alternatively, high-throughput DNA sequencing of the genome of vHVT013-69 may be employed to definitively characterise genome similarity and identify any sequence differences compared to the wild type HVT. The changes in genome function as a result of the insertion of this transgene are difficult to predict, but may include important factors such as host range that have not been directly addressed in this study (i.e. does the HVT of the vHVT013-69 vaccine, have increased host range compared to wild type HVT?).

Murid (murine) herpesvirus 1 principle gene A promoter is part of the transgenic cassette. The effects of this promoter on global gene expression of the HBV virus have also not been studied by the applicant. There are also other changes in the HBV virus genome that do not receive any biosafety assessment. The applicant states that during the insertion of the transgenic cassette a small part of the genome (15bp) was deleted, but this genetic change is not featured in the biosafety assessment by the applicant. Recent findings also indicate that the viral RNA-dependent RNA polymerase have the ability to recognize heterologous 3´ UTRs (from the lettuce mosaic virus and the cucumber mosaic virus) included in transgene mRNAs, and to use them as transcription promoters (Jones *et al*. 1993 and 1996). These findings have important implications for the safety of viral resistant transgenic plants in general since they indicate changes in intergenic or UTR regions can lead to activation of other genes and viruses.

Risk of transfer to other species

Since the virus used is a live and not the attenuated vaccine as used in the past (i.e. attenuated MDV1, attMDV1 is virus that is heat or chemical treated so that the virus can no longer replicate but still generate an immune response) there are increased risks of viral escape since the live virus can replicate in the host, thereby increasing in number. Furthermore, the close relatedness between pathogenic and non-pathogenic strains of the MDV serotypes increases the risks that even small mutations or recombination events of the vHVT013-69 virus could result in pathogenicity.

It was noted that experiments carried out by the applicant demonstrate that transmission between young chickens does not occur. However, similar experiments by the applicant established that the transmission from chicken to turkey does occur. "..turkeys were reared in contact with chickens vaccinated with an overdose of either the parental or the recombinant strain. Examination demonstrated that both strains were transmitted to turkeys in these conditions". Therefore if the virus does cross to turkey then transmission and dissemination amongst the turkey population would occur. The applicant provides evidence that transmission between turkeys does occur, but states, "It is likely that the spread of the recombinant strain would be limited in turkey populations that are very frequently infected by HVT". Since HVT is very prevalent-"prevalence in turkeys approaches 100%" with many carriers of the disease (nonpathogenic) (section 16 of risk assessment) the spread of vHVT013-69 in the environment amongst turkeys will be

complete and rapid. Furthermore, this will not be limited to turkey since HVT infects many Galliforms and other birds (quail, pheasant, pigeon or goose). The applicant's results showed that both chickens and turkeys were infected by the vaccine with a low dose injected subcutaneously. However, when the vaccine was not injected but dispersed in the bedding/litter; chickens were not infected whereas turkeys were partially infected by this route of transmission. These results help explain why turkeys transmit the disease more easily than chickens, but the lack of transmission of HVT in chickens is at least partly dependent upon age. Studies have shown that transmission does occur when the chickens are older than 8 weeks (Cho, 1975). The conclusions from the applicant of lack of transmission in chickens are not substantiated since the studies seem to be limited to chickens 28 day old.

Furthermore, although HVT is thought to only infect avian, viral members of this family infect humans (i.e. virus from the family Herpesviridae infect human causing human Herpes, chickenpox and mononucleosis) (Gompels *et al.* 1995). There are uncertainties in extent of the current host range as well as the future host range (the live vaccine which replicates in the host) due to molecular evolution of viruses and viral recombination. In any case, there seems to high probability that the live vHVT013-69 virus will not be contained in chicken and can escape to infect other species. These risks depend on the consequence or detriment since the virus main remain non-pathogenic in the new host species. The switch from pathogenic to non-pathogenic virus is poorly understood and viruses are rapidly evolving and there is the possibility of recombination to form new pathogenic viruses.

Viral recombination and creation of new viruses

Adaptation of living organisms to a changing environment through evolution requires a compromise between genetic variation and phenotypic selection. Viruses, and particularly RNA viruses, have a high variability that is thought to be due to three main evolutionary forces- mutation, reassortment and recombination (Roossinck, 1997; Domingo & Holland, 1997). Viral RNA replication is characterized by a high mutation rate, due to the lack of proofreading-repair of viral RNA-dependent RNA polymerases. This, in conjunction with short replication times and a high multiplicity, leads towards a dynamic mutant population, termed virus quasi-species that correspond to a swarm of sequence variants (Holland & Domingo, 1998). The genetic divergence is restricted by the necessity to maintain a functional viral RNA genome and by environmental selective pressures. By allowing the spread of new mutations, both reassortment and recombination increase genetic variability and favour the creation of variant viruses that may be best adapted to withstand future environmental selective pressure on the virus population (Lai, 1992; Carpenter & Simon, 1996).

The risks of transgene/virus recombination can only be understood in the context of the background level of recombination between viruses in natural

settings. However, new, successful (and more pathogenic) variants of viruses do arise naturally by recombination (Chenault and Melcher 1994; Revers *et al* 1996; Padidam *et al* 1999). In nature, the majority of new viruses arising as a result of recombination is nonviable or has low fitness. Success of a given variant depends upon the conditions and selective pressures. The most relevant here is the chicken hosts immune response that continuously selects viral variants for increased fitness or virulence posing risks for the development of new viruses with the ability to introduce new diseases.

Questionable benefits

The comparisons have not be clearly made to establish any real scientific benefits of using a GM virus as opposed the native/natural viruses: HVT + IBD or the attenuated MDV1 + IBD. There does seem to be some negative effect of using HVT + IBD with immunosuppressive effects and consequent morbidity and mortality, but no data compares the two and in particular those made with attenuated MDV1 + IBD. The comparisons of efficacy and biosafety need to be carried out so that the benefits of using this GM live vaccine can be weighed against the biosafety risks.

With high-intensity breeding, large-scale closed rearing of birds, and increased global shipping of poultry, epidemic disease becomes a major concern. The appropriateness of these high technology methods for South Africa should also form part of the assessment since a large proportion of agricultural practice does not raise chickens in isolated houses but may range outdoors where there will be interactions with other avian species. Importantly, the biosafety assessment is limited to young chickens (generally less than 1 month old) in intensive chicken houses in other countries (not South Africa).

In summary, adhering to the precautionary principle indicates that there are considerable biosafety risks that have not been adequately addressed by the applicant and therefore vHVT013-69 should not currently be approved for general release.

References

Bao, P.H.; Castiglione, S.; Giordani, C.; Li, W.; Wang, G.; Datta, S.K.; Datta, K.; Potrykus, I.; Sala, F. (1993). State of the foreign gene and of the genome in transgenic rice (Orvza sativa L.). Cytotechnology 11: S123125,

Becker Y, Tabor E, Asher Y, Davidson I, Malkinson M, Witter R L. PCR detection of amplified 132 bp repeats in Marek's disease virus type 1 (MDV-1) DNA can serve as an indicator for critical genomic rearrangement leading to the attenuation of virus virulence. Virus Genes. 1993;7:277–287.

Biggs, P.M., Payne, L.N., Miline, B.S., Churchill, A.E., Chubb, R.C., Powell, D.G. and Harris, A.H., 1970. Field trials with an attenuated cell associated vaccine for Marek's disease. Vet. Rec. 87, pp. 704–709.

Buckmaster A E, Scott S D, Sanderson M J, Boursnell M E G, Ross N L J, Binns M M. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implications for herpesvirus classification. J Gen Virol. 1988;69:2033–2042.

Cao,Y. C.; W. S. Yeung, M. Law, Y. Z. Bi, F. C. Leung, B. L. Lim Molecular Characterization of Seven Chinese Isolates of Infectious Bursal Disease Virus: Classical, Very Virulent, and Variant Strains. Avian Diseases, Vol. 42, No. 2 (Apr. - Jun., 1998), pp. 340-351doi:10.2307/1592484

Cho B R, Kenzy S G. Horizontal transmission of turkey herpes virus to chickens. III. Transmission in three different lines of chickens. Poult Sci. 1975; 54:109–115.

Cho B R. Horizontal transmission of turkey herpes virus to chickens. IV. Viral maturation in the feather follicle epithelium. Avian Dis. 1974; 19:136–141.

Cho, B. R. (1975) Horizontal Transmission of Turkey Herpes virus to Chickens IV. Viral Maturation in the Feather Follicle Epithelium Avian Diseases, Vol. 19, No. 1 pp. 136-141. Doi: 10.2307/1588964

Cho, B.R., 1977. Dual virus maturation of both pathogenic and apathogenic Marek's disease herpes virus (MDHV) in the feather follicles of dually infected chickens. Avian Dis. 21, pp. 501–507.

Churchill, A.E. and Biggs, P.M., 1967. Agent of Marek's disease in tissue culture. Nature 215, pp. 528–530.

Churchill, A.E., Payne, L.N. and Chubb, R.C., 1969. Immunisation against Marek's disease using a live attenuated virus. Nature 221, pp. 744–747.

Darteil R, Bublot M, Laplace E, Bouquet J-F, Audonnet J-C, Riviere M. Herpesvirus of turkey recombinant viruses expressing infectious bursal disease virus (IBDV) VP2 immunogen induce protection against an IBDV virulent challenge in chickens. Virology. 1995; 211:481–490.

Fabricant J, Calnek B W, Schat K A. The early pathogenesis of turkey herpes virus infection in chickens and turkeys. Avian Dis. 1982; 26:257–264.

Fukuchi K, Tanaka A, Schierman L W, Witter R L, Nonoyama M. The structure of Marek's disease virus DNA: the presence of unique expansion in nonpathogenic viral DNA. Proc Natl Acad Sci USA. 1985; 82:751–754.

Gompels U A, Nicholas J, Lawrence G, Jones M, Thomson B J, Martin M E D, Efstathiou S, Craxton M, Macaulay H A. The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. Virology. 1995;209:29–51.

Green, A.E. and Allison, R.F. (1994). Recombination between viral RNA and transgenic plant transcripts. Science 263, 1423.

Greene A.E. and Allison, R.F. (1996). Deletions in the 3' untranslated region of cowpea chlorotic mottle virus transgene reduce recovery of recombinant viruses in transgenic plants. Virology 225, 231-4.

Handberg, K.J., Nielsen, O.L. and Jorgensen, P.H., 2001. The use of serotype 1- and serotype 3-specific polymerase chain reaction for the detection of Marek's disease virus in chickens. Avian Pathol. 30, pp. 243–249.

Infectious Bursal Disease: The Merck Veterinary Manual (2006). Retrieved on 2007-06-26. ICTVdB Management (2006). 00.009.0.02.001. Infectious bursal disease virus. In: ICTVdB - The Universal Virus Database, version 4. Büchen-Osmond, C. (Ed), Columbia University, New York, USA [1]

Islam, A.F.M., Walkden-Brown, S.W., Burgess, S.K. and Groves, P.J., 2001. Marek's disease in broiler chickens: Avian Pathol. 30, pp. 621–628.

Izumiya, Y., Jang, H.-K., Ono, M. and Mikami, T., 2001. A complete genomic DNA sequence of Marek's disease virus type 2, strain HPRS24. Curr. Top. Micrbiol. Immunol. 255, pp. 191–221.

Jones D, Brunovskis P, Witter R, Kung H-J. Retroviral insertional activation in a herpesvirus: transcriptional activation of US genes by an integrated long terminal repeat in a Marek's disease virus clone. J Virol. 1996;70:2460–2467.

Jones D, Isfort R, Witter R, Kost R, Kung H-J. Retroviral insertions into a herpesvirus are clustered at the junctions of the short repeat and short unique sequences. Proc Natl Acad Sci USA. 1993;90:3855–3859.

Kasanga C.J. T. Yamaguchi, P. N. Wambura, A. D. Maeda-Machang'u, K. Ohya and H. Fukushi (2007) Molecular characterization of infectious bursal disease virus (IBDV): Diversity of very virulent IBDV in Tanzania. Archives of Virology Volume 152, Number 4 / April, 2007 Pages 783-790

Kingham, B.F., Zelnik, V., Kopacek, J., Majerciak, V. and Schmidt, C.J., 2001. The genome of herpes virus of turkeys: comparative analysis with Marek's disease viruses. J. Gen. Virol. 82, pp. 1123–1135.

Kingham, et al. (2001) The genome of herpesvirus of turkeys: comparative analysis with Marek's disease virusesJournal of General Virology, 82, 1123–1135. Printed in Great Britain and Carl J. Schmidt1

Kishi M, Harada H, Takahashi M, Tanaka A, Hayashi M, Nonoyama M, Josephs S F, Buchbinder A, Schachter F, Ablashi D V, Wong-Staal F, Salahuddin S Z, Gallo R C. A repeat sequence, GGGTTA, is shared by DNA of human herpesvirus 6 and Marek's disease virus. J Virol. 1988;62:4824–4827.

Kyle S. MacLea and Hans H. Chen (2007) The Threat of Marek's DiseaseVirus Is Expanding. Microbe / Volume 2, Number 5, 2007

Lee K. Long term effects of Marek's disease vaccination with cell-free herpesvirus of turkey and age at debeaking on performance and mortality of white leghorns. Poult Sci. 1980;59:2002–2007.

Lee L F, Wu P, Sui D, Ren D, Kamil J, Kung H J, Witter R L. The complete unique long sequence and the overall genomic organization of the GA strain of Marek's disease virus. Proc Natl Acad Sci USA. 2000;97:6091–6096.

Lee, L.F., Wu, P., Sui, D., Ren, D., Kung, H.J. and Witter, R.L., 2000. The complete unique long sequence and the overall genomic organization of the GA strain of Marek's disease virus. Proc. Natl. Acad. Sci. USA 97, pp. 6091–6096.

Nazerian, K., Solomon, J.J., Witter, R.L. and Burmester, B.R., 1968. Studies on the etiology of Marek's disease. II. Finding of a herpes virus in a cell culture. Proc. Exp. Biol. Med. 127, pp. 177–182...

Parcells M S, Anderson A S, Cantello J L, Morgan R W. Characterization of Marek's disease virus insertion and deletion mutants that lack US1 (ICP22 homolog), US10,

and/or US2 and neighboring short-component open reading frames. J Virol. 1994;68:8239–8253.

Pinkel D, and Albertson D.G. (2005). Comparative genome hybridization. Annual Review of Genomics and Human Genetics, Vol. 6, Pages 331-354

Purchase H G, Okazaki W, Burmester B R. Long-term field trials with the herpesvirus of turkey's vaccine against Marek's disease. Avian Dis. 1972;16:57–71.

Reddy S K, Sharma J M, Ahmad J, Reddy D N, McMillen J K, Cook S M, Wild M A, Schwartz R D. 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. 1996;14:469–477.

Ross N, Binns M M, Sanderson M, Schat K A. Alterations in DNA sequence and RNA transcription of the BamHI-H fragment accompany attenuation of oncogenic Marek's disease herpesvirus. Virus Genes. 1993;7:33–51.

Santin Elizabeth R. ; Shamblin, Christine E. ; Prigge, Jonathan T. ; Arumugaswami, Vaithilingaraja ; Dienglewicz, Robert L. ; Parcells, Mark s (2006) . Avian diseases (avian dis.) issn 0005-2086. vol. 50, no1, pp. 96-103.

Schoelz, J.E. and Wintermantel, W.M. (1993). Expansion of viral host range through complementation and recombination in transgenic plants. Plant Cell 5, 1669-79.

Sharma J M, Burmester B R. Resistance to Marek's disease at hatching in chickens vaccinated as embryos with the turkey herpesvirus. Avian Dis. 1982;26:134–149.

Sharma J M, Lee L F, Wakenell P S. Comparative viral, immunologic, and pathologic responses of chickens inoculated with herpesvirus of turkeys as embryos or at hatch. Am J Vet Res. 1984;45:1619–1623.

Sharma J M. Delayed replication of Marek's disease virus following in ovo inoculation during late stages of embryonal development. Avian Dis. 1987;31:570–576.

Sheppard M, Werner W, Tsatas E, McCoy R, Prowse S, Johnson M. Fowl adenovirus recombinant expressing VP2 of infectious bursal disease virus induces protective immunity against bursal disease. Arch Virol. 1998;143:915–930.

Silva R F, Witter R L. Genomic expansion of Marek's disease virus DNA is associated with serial in vitro passage. J Virol. 1985;54:690–696.

Tulman E R, Afonso C L, Lu Z, Zsak L, Rock D L, Kutish G F. The genome of a very virulent Marek's disease virus. J Virol. 2000;74:7980–7988.

Walkden-Brown, S.W., Groves, P.J., Islam, A., Rubite, A., Islam, A.F.M.F. and Burgess, S.K., 2004. Monitoring Marek's disease virus in broiler flocks using standard and real-time quantitative PCR of spleen and dust samples. Proc. Aust. Poult. Sci. Symp. 16, pp. 149–152.

Walkden-Brown, S.W., Groves, P.J., Islam, A.F.M.F., Burgess, S.K., Arzey, K.E., Mascord, L. and Young, P.L., 2003. Differentiation of Marek's disease virus serotypes using PCR: research and field experience. Proc. Aust. Poult. Sci. Sym. 15, pp. 192–196.

Wei, P. and Cui, Z., 2002. The comparison of Meq gene sequences amplified from different pathotypes of Marek's disease virus. Chin. J. Prev. Vet. Med. 24, pp. 88–92.

Wintermantel, W.M. and Schoelz, J.E. (1996). Isolation of recombinant viruses between cauliflower mosaic virus and a viral gene in transgenic plants under conditions of moderate selection pressure. Virology 223, 156-64.

Witter R L, Nazerian K, Purchase H G, Burgoyne G H. Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. Am J Vet Res. 1970;31:525–538.

Witter R L, Solomon J J. Experimental infection of turkeys and chickens with a herpesvirus of turkeys (HVT). Avian Dis. 1972;16:34–44. [

Witter R L. Increased virulence of Marek's disease virus field isolates. Avian Dis. 1997;41:149–163.

Witter R L. Turkey herpesvirus: lack of oncogenicity for turkeys. Avian Dis. 1972;16:666–670.

Witter, R.L., 2001. Protective efficacy of Marek’s disease vaccines. Curr. Top. Micrbiol. Immunol. 255, pp. 57–90.

Zelnik V, Darteil R, Audonnet J C, Smith G D, Riviere M, Pastorek J, Ross L J N. The complete sequence and gene organization of the short unique region of herpesvirus of turkeys. J Gen Virol. 1993;74:2151–2162

Zelnik V, Ross N L J, Pastorek J. Characterization of proteins encoded by the short unique region of herpesvirus of turkey's by in vitro expression. J Gen Virol. 1994;75:2747–2753.