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Ian Tarpey

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Expression of chicken Interleukin-2 by turkey herpesvirus increases the immune response against Marek's disease virus but fails to increase protection against virulent challenge

**I.Tarpey<sup>1</sup>, P.J.Davis<sup>1</sup>, P.Sondermeijer<sup>2</sup>, C. van Geffen<sup>2</sup>, I Verstegen<sup>2</sup>, V.E.J.C. Schijns<sup>2</sup>, Jill Kolodsick<sup>3</sup> and R. Sundick<sup>3</sup>.**

Intervet UK, Walton Manor, Walton, Milton Keynes, Bucks. UK. MK7 7AJ.<sup>1</sup>

Intervet International, PO Box 31, Boxmeer, Netherlands 5830.<sup>2</sup> Wayne State University, Detroit, Michigan MI 48201.<sup>3</sup>

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Corresponding author

Tel: 00 44 (0)1908 658206

Fax: 00 44 (0)1908 685623

E-mail: [ian.tarpey@intervet.com](mailto:ian.tarpey@intervet.com)

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**Expression of chicken Interleukin-2 by turkey herpesvirus increases the immune response against Marek's disease virus but fails to increase protection against virulent challenge**

**I.Tarpey<sup>1</sup>, P.J.Davis<sup>1</sup>, P.Sondermeijer<sup>2</sup>, C. van Geffen<sup>2</sup>, I Verstegen<sup>2</sup>, V.E.J.C. Schijns<sup>2</sup>, Jill Kolodsick<sup>3</sup> and R. Sundick<sup>3</sup>.**

**Abstract.**

As Marek's disease virus continues to evolve towards greater virulence more efficacious vaccines will be required in the future. We expressed chicken interleukin-2 (IL-2) from a turkey herpesvirus (HVT) in an attempt to increase the efficacy of HVT as a vaccine against Marek's disease. The recombinant IL-2/HVT was safe for *in ovo* vaccination though it replicated less in the birds compared to the parent HVT strain. Expression of IL-2 increased the neutralizing antibody response against HVT but did not increase the protection against virulent Marek's disease virus challenge.

## Introduction

Marek's disease virus (MDV) is an alphaherpesvirus which causes lymphoproliferative disorders in domestic chickens and is characterized by mononuclear cellular infiltrates in many organs and in peripheral nerves. Three serotypes of MDV have been identified, and all oncogenic strains belong to serotype 1. Infection of chickens with virulent virus is thought to take place via the respiratory route, following the shedding of virus into poultry house from previously infected chickens. The virus sets up cytolytic infections in B and T cells, followed by latent infections in T cells. In genetically susceptible birds, CD4+ T cells can become transformed and lymphomas may develop later in a number of different tissues.

Current MDV vaccines consist of either attenuated serotype 1, naturally apathogenic serotype 2, or serotype 3 strain isolated from turkeys (HVT), or mixtures thereof. Together these vaccines have been remarkably successful in the reduction in the number of cases of Marek's disease (MD) worldwide since their introduction in the early 1970s (Witter, 2001). Vaccine induced protection is not well understood but may involve both innate and adaptive immunity, and the induction of cellular responses including natural killer (NK) cells (Sharma, 1981, Heller and Schat, 1987), macrophages (Gupta et al., 1989) and Marek's disease-specific cytotoxic T lymphocytes (CTLs) (Omar and Schat, 1996). The role of antibodies in protection is unclear, though neutralising maternally derived antibody is known to delay the replication of both vaccinal and challenge virus (Chubb & Churchill, 1969; Calnek & Smith, 1972). Many chicken cytokines and chemokines have now been cloned (Leutz et al., 1989; Digby & Lowenthal, 1995; Weining et al., 1996; Sundick & Gill-Dixon,

1997; Schneider et al., 2000; Schneider et al., 2001; Balu & Kaiser, 2003; Avery et al., 2004; Degen et al., 2004; Rothwell et al., 2004; Santos et al., 2006) and the role of these immunomodulators in resistance to MD can now be elucidated. Thus far, chicken granulocyte colony stimulating factor (originally identified as myelomonocytic growth factor) and IFN- $\gamma$  (including IFN- $\gamma$ -induced nitric oxide) have been shown to be potentially important in disease resistance (Sharma, 1989, Xing & Schat, 2000, Djebara et al., 2002, Santos et al., 2006).

Whilst vaccines have dramatically reduced the number of cases of MD, there has been a constant evolution of the serotype 1 MDV isolates towards greater virulence (Witter, 1997). Current field viruses are able to cause significant levels of MD in vaccinated birds, and this increase in virulence has prompted a number of groups to develop recombinant MD vaccines in an effort to improve vaccine efficacy (Nazerian et al., 1992; Morgan et al., 1998; Lee et al., 2003; Baigent et al., 2006). Though some of the recombinant MD vaccines have provided good levels of protection against MDV in laboratory trials, none are commercially available for widespread use.

Interleukin-2 (IL-2) is an important signaling molecule in most mammalian immune responses stimulating the proliferation of T cells and NK cells (Ortaldo et al., 1984; London et al., 1986; Talmadge et al., 1986). Since the cloning of chicken IL-2 (Sundick & Gill-Dixon, 1997) several studies have demonstrated that this cytokine shares some of the properties of its mammalian counterpart (Choi & Lillehoj, 2000, Hilton et al., 2002, Hulse et al., 2004, Kogut et al., 2002, Gobel et al., 2003) whilst also promoting growth of chickens following *in ovo* inoculation (Ford et al., 2002). Mammalian IL-2 also plays a critical role in the development and expansion of regulatory T cells (Nelson, 2004), though it is unknown whether chicken IL-2 has a

similar function. We examined whether *in ovo* vaccination with a recombinant HVT expressing chicken IL-2 was safe, would promote growth and/or modulate the immune response against MDV thereby increasing the efficacy of the HVT vaccine against a virulent MD challenge.

## Materials and Methods

**Chickens.** Specified Pathogen Free (SPF) White Leghorn chicken embryonated eggs (Lohmann, Germany) were used in all experiments. These birds are of a genotype that is moderately susceptible to MD (Marcus Koch, Lohmann Tierzucht, personal communication). All chicks were hatched in a contained environment and transferred to negative pressure isolators or floor pens for the remainder of the experiments. Chick starter crumbs and water were provided during the course of the experiments.

**Cells and viruses.** Primary chick embryo fibroblasts (CEFs) were prepared by trypsinising 11 day old specific pathogen free (SPF) embryos (Lohmann) until single cells were obtained. Secondary CEFs were prepared by trypsinisation of primary CEFs monolayers. Dermal cells for DNA transfection were prepared by trypsinising 11 day old embryos for 5 minutes. Cells were maintained in M6B8 medium (Invitrogen) containing 5% foetal calf serum (Sigma) in a humidified atmosphere of 5% CO<sub>2</sub> at 37<sup>0</sup>C. Recombinant HVT stocks were prepared as described below. The HVT PB-1 strain was used as the parent strain for preparation of the recombinant HVT. RB1B virus passaged 12 times on chick kidney, back-passaged twice through SPF White Leghorns, then passaged a further five times on chick kidney cells, was

used as the virulent challenge virus. All viruses were diluted in Nobilis Diluent CA © (Intervet).

**Transfer plasmid construction.** The transfer plasmid pVEC38 is based on the pVEC04 plasmid (Sondermeijer et al., 1993) enabling insertion of genes into the US10 site of HVT. pVEC38 is identical to pVEC04 except that protein expression is driven by the pseudorabies virus gB promoter. The chicken IL-2 gene was amplified by the polymerase chain reaction using Pfu polymerase (Stratagene) as a blunt ended fragment with the oligonucleotides TGGGACACTGCCATGATGTGCAAA and TTATTTTTGCAGATATCTCACAAA. The IL-2 fragment was treated with T4 DNA kinase (New England Biolabs) and ligated into pVEC38 that had been digested with *Bgl*III, blunt-ended with T4 DNA polymerase (New England Biolabs) and treated with calf intestinal alkaline phosphatase (New England Biolabs). The inserted gene and flanking regions were sequenced.

**Preparation of recombinant HVT.** The pVEC38 transfer plasmid containing the chicken IL-2 gene was transfected into chick embryo dermal cells along with 10µg of HVT PB-1 DNA prepared as previously described (Morgan et al., 1990). The cells were passaged and single virus plaques picked under an agar overlay. The viruses were sonicated, re-plated, plaque picked then passaged a further four times on primary CEFs. Plaques were probed after 5 days of incubation on secondary CEFs by removing the agar overlay and making duplicate nylon filter lifts from the cells. One filter was probed for the presence of IL-2 and the other for HVT DNA by hybridization using the random primer enhanced chemiluminescence system



(Amersham). Alignment of the filters confirmed the presence of IL-2 in each HVT plaque.

**Characterisation of the IL-2/HVT recombinant.** To monitor growth *in vitro* primary CEFs were infected with the recombinants at a multiplicity of infection (MOI) of 0.01 and harvested at 24, 48 or 72 hours. The viruses were assayed on secondary CEF. To monitor IL-2 expression *in vitro*, supernatant from infected CEFs was harvested at 2 days post incubation. Bioassays for chicken IL-2 were performed as described (Sundick & Gill-Dixon, 1997).

Viraemia was assessed in groups of 20 SPF embryos vaccinated with 1,000 plaque forming units (pfu) of IL-2/HVT. Eggs on the 18<sup>th</sup> day of embryo development were inoculated into the amniotic fluid with 0.1ml of virus using a 20 gauge, 2.5cm long needle. The birds were bled 14 days post hatch, a time known to coincide with the peak viraemia level of the HVT PB-1 parent strain (I. Tarpey, unpublished observations). Birds were also bled at 28 days post hatch. Peripheral blood leucocytes (PBL) were collected over Ficoll (Pharmacia), counted and  $2 \times 10^7$  cells added to secondary CEFs in 100mm dishes. After 48 hours the media were collected for IL-2 bioassay and the plates overlaid with an agar/media mix. After 5 days the plaques were counted and probed in duplicate for the presence of IL-2 or HVT confirming the absence of wild type HVT. Viraemia levels were expressed as pfu per number of PBL.

**Safety of the IL-2/HVT for *in ovo* vaccination.** To monitor vaccine safety 50 eighteen-day old SPF White Leghorns embryos were vaccinated with up to 10,000 pfu

of IL-2/HVT via the amniotic route. The chicks were hatched in separate hatching units, moved to floor pens and the general health monitored for up to 6 weeks.

**Antibody response and weight gain in birds vaccinated with IL-2/HVT.** To analyse the antibody response induced by IL-2/HVT 20 eighteen day old SPF White Leghorns embryos were vaccinated via the amniotic route with 1000 pfu. The birds were bled and sera collected 3, 5 and 7 weeks post vaccination. Neutralising antibody titres were analysed as described previously (Churchill et al., 1973). To monitor weight gain, groups of 100 embryos were vaccinated *in ovo* with 1000pfu and allowed to hatch. A defined amount of food was provided each day and any remaining food was weighed. Feed conversion was monitored throughout the experiment and at 6 weeks the birds were sexed and weighed.

**Efficacy of the IL-2/HVT recombinant vaccine.** In the efficacy trials, groups of 100 SPF embryos were inoculated with 1,000 pfu of IL-2/HVT via the amniotic route, allowed to hatch then challenged via the intramuscular route with 500 pfu RB1B five days post hatch. The birds were observed for clinical signs of MD for 10 weeks. Dead birds, and birds that were euthanased for humane reasons, were examined for macroscopic tumours. If no tumours were found in these birds, and also not in those birds euthanised at the end of the study, their tissues (spleen, heart, liver, kidneys, bursa, gonads and sciatic nerves) were taken for histological examination for microscopic lesions. Vaccine efficacy is expressed as the protective index:

$$\text{Protective index} = \frac{\% \text{MD non-vaccinated controls} - \% \text{MD test groups}}{\% \text{MD non-vaccinated controls}} \times 100$$

**Statistical analysis.** A two-tailed T test was used to compare the serological response. P-values of less than 0.05 were considered to be significant.

## Results

**Cloning and characterisation of the recombinant IL-2/HVT.** Thirty viral plaques that had been passaged a total of six times were found to contain a single copy of the IL-2 gene by hybridization and two were randomly selected for further analysis. Analysis by probing of at least 500 plaques in duplicate for the presence of IL-2 and HVT sequences showed that these viruses did not contain any wild type HVT. The recombinant viruses had similar growth kinetics to the parent HVT *in vitro* (Figure 1A). Analysis of tissue culture supernatants showed that these viruses expressed bioactive IL-2 *in vitro* (Figure 1B). Following *in ovo* inoculation the two recombinant viruses could be re-isolated from PBL on day 14 post hatch at an average 4 fold lower rate than the parent HVT strain (Figure 1C) and both maintained IL-2 expression after re-isolation (Figure 1D). The recombinants could also be re-isolated on day 28 post hatch though were not quantitated (results not shown). Analysis by duplicate probing for the presence of IL-2 and HVT sequences showed that viruses re-isolated at both day 14 and 28 post vaccination remained 100% positive for the presence of the IL-2 gene and no wild type HVT was detected. One of the viruses (IL-2/HVTA) was chosen for further study.

**Safety of IL-2/HVT for *in ovo* vaccination.** As mammalian IL-2 is an important regulator of immune responses, it was necessary to determine if potential expression of IL-2 *in ovo* and prolonged expression in the chicken via the viraemia of the IL-

2/HVT, was safe. For this purpose, 18-day old embryos were vaccinated with a higher dose of IL-2/HVT. As shown in Figure 2, the hatchability of the embryos vaccinated with 10,000pfu of IL-2/HVT was almost identical to embryos vaccinated with 1,000pfu of either the parent strain or IL-2/HVT and to non-vaccinated embryos. Furthermore, the hatched chickens, kept for 6 weeks, did not show any clinical signs of MD or any morbidity that could be associated with over-expression of IL-2. These results suggest that the IL-2/HVT recombinant is safe for *in ovo* vaccination.

**IL-2 expression fails to increase weight gain in vaccinated chicks.** Delivery of IL-2 *in ovo* has previously been shown to increase weight gain in hatched chicks. To determine if this result could be confirmed using HVT to deliver IL-2 *in ovo*, embryos were vaccinated with IL-2/HVT. Chicks were weighed at hatch and weekly until 6 weeks. Feed conversion was monitored throughout the experiment. Typical results indicating that vaccination with IL-2/HVT does not significantly increase weight gain in comparison to HVT vaccination in sex matched chicks over the 6 week period are shown in Figure 3. Feed conversion was also unaffected (data not shown).

**IL-2 expression increases the neutralising antibody response against HVT**

To determine if IL-2 expression could increase the antibody response against HVT, two groups of embryos were vaccinated with 1000pfu of either IL-2/HVT or HVT. The birds were bled at 3, 5 and 7 weeks of age. Vaccination with IL-2/HVT significantly increased the neutralising antibody response against HVT ( $p=0.02$ ) at 7 weeks post vaccination (Figure 4) though not at 3 or 5 weeks.

**IL-2/HVT fails to increase protection against virulent MDV challenge.** The efficacy of the recombinant vaccine was assessed in three separate experiments. In each of the experiments IL-2/HVT failed to improve the efficacy of the vaccine in comparison to the HVT parent. Table 1 shows typical results with the IL-2/HVT inducing 43% protection compared to 46% with the parent HVT strain. There were no significant differences in the clinical outcome of the challenges based on the number of macroscopic or microscopic tumours detected. In all of the experiments the non-vaccinated control birds died within 5-7 weeks of challenge.

## Discussion

We have investigated the use of a recombinant HVT expressing chicken IL-2 as a vaccine against MD. The recombinant virus showed similar growth kinetics *in vitro* as the parent HVT strain and expressed bioactive cytokine suggesting that insertion of the gene and protein expression did not disable the virus. Passage of the virus in culture showed that the IL-2 gene could be stably maintained by the virus and that the recombinants were free of wild type HVT. However, the viraemia studies demonstrated that at 14 days post hatch less IL-2/HVT virus could be re-isolated from the birds in comparison to the parent HVT strain. The reason for this result is unknown though it could be due to a more rapid clearance of the virus by the immune system or that the IL-2/HVT may have reached peak levels of replication in PBL earlier than the parent HVT strain. However we cannot rule out that insertion of the IL-2 gene had a disabling effect on the replication of the IL-2/HVT *in vivo* as this has been observed with other HVT recombinants that grow normally in tissue culture (I. Tarpey, unpublished observations). Importantly, the re-isolated recombinant viruses

had maintained the IL-2 gene and expressed bioactive interleukin-2 suggesting that these viruses were secreting the cytokine *in vivo*. It would be interesting to determine the replication kinetics of the IL-2/HVT recombinant viruses in maternally derived antibody (MDA) positive birds, as MDA is known to affect the replication of HVT (Calnek and Smith, 1972).

On the basis of hatchability and the lack of morbidity post hatch, the results suggest that the IL-2/HVT was safe for delivery *in ovo*, the slight reduction in hatch in comparison the parent strain most likely being associated with the use of separate hatchers. However, as mammalian IL-2 can promote the proliferation of T and NK cells but under circumstances can also down-regulate T cell responses (Nelson, 2004), future work will analyse the *in vivo* effects of chicken IL-2 expression from HVT at a cellular level.

Weight gain studies demonstrated that vaccination with IL-2/HVT failed to promote growth or increase feed conversion in comparison to vaccination with HVT. Previous studies have shown that *in ovo* inoculation of IL-2 could increase weight gain when a defined amount of IL-2 was delivered (Ford et al, 2002). It is unknown whether IL-2 was expressed in the embryo prior to hatch though this could be expected as HVT has been shown to grow in the embryo following *in ovo* vaccination (Sharma, 1987). However it is not possible to estimate the amount of IL-2 being expressed in the embryo and for this reason it may be worthwhile to investigate whether higher doses of IL-2/HVT *in ovo* can promote weight gain.

Vaccination with IL-2/HVT significantly increased the neutralizing antibody response to HVT seven weeks but not three or five weeks after hatch. This result suggests that chicken IL-2 can directly, or indirectly via T cell help, modulate the B cell response in IL-2/HVT vaccinated chickens in line with data demonstrating that

IL-2 can increase the IBDV-specific antibody via plasmid DNA vaccination (Li et al 2004, Yu et al., 2001). In addition this result provides evidence that prolonged expression of IL-2 does not perturb the immune response. It is unclear why the increase in antibody response induced by IL-2/HVT was not detected before seven weeks though this may be associated with the possible delay in replication of the IL-2/HVT recombinant virus. It would be of interest to determine the duration of the increased antibody response and in particular, whether vaccinated breeder birds could pass on higher levels of maternally derived antibody (MDA) to progeny. Furthermore, as MD vaccines are routinely co-delivered with IBDV and Fowlpox vaccines (Ricks et al., 1999) it would be interesting to determine if the IL-2 expression augments the response to co-delivered vaccines.

The IL-2/HVT recombinant provided slightly lower protection against virulent MDV challenge than the parent HVT strain. Due to age resistance MD challenge takes place early after hatch and in this model there may have been insufficient time for adequate IL-2 expression to affect the outcome of the challenge. In agreement with this, the late increase in antibody levels associated with IL-2 expression was unlikely to afford protection, an early cellular immune response being more likely to improve protection. It can be speculated that the slightly lower protection is associated with the lower replication of the recombinant virus though this would need to be confirmed experimentally. Regardless, IL-2 expression could not overcome the lower replication of the recombinant to increase efficacy in comparison to the parent strain. As an alternative to this challenge model it would be of interest to study viral load to determine if the IL-2/HVT recombinant virus could reduce the amount of virulent MDV post challenge. Furthermore, as the birds used in these studies were moderately susceptible to MD, it would be interesting to test the efficacy of the IL-

2/HVT using inbred fully genetically susceptible birds. Future experiments will determine whether cytokines such as IL-12, IFN- $\gamma$  or IL-18, more associated with the induction of cell mediated immunity, would improve the protection against virulent MD challenge when expressed by HVT.

This is the first example of HVT being used to deliver a chicken cytokine. These studies have shown that chicken IL-2 can be stably expressed by HVT, can augment the immune response against MDV but does not improve the efficacy of HVT in this MD challenge model.



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Table 1. Efficacy of IL-2/HVT against a virulent MD challenge.

Groups of 100 18 day old embryos were vaccinated via the amniotic route with 1000pfu of virus. Hatched chicks were challenged at 5 days of age with 100pfu of virulent RB1B via the intramuscular route along with non-vaccinated, age matched chicks. The clinical outcome of Marek's disease challenge and the protective index are presented.

| Clinical outcome   | Treatment group |     |                         |
|--|-----------------|-----|-------------------------|
|  | IL-2/HVT        | HVT | Non-vaccinated controls |
| Death or euthanasia during study<br>Macroscopic lesions detected | 37              | 35  | 77                      |
| Death or euthanasia during study<br>Microscopic lesions detected | 4               | 6   | 23                      |
| Macroscopic lesions detected at the<br>end of the study          | 11              | 10  | 0                       |
| Microscopic lesions detected at the<br>end of the study          | 5               | 3   | 0                       |
| Protective index   | 43              | 46  | 0                       |

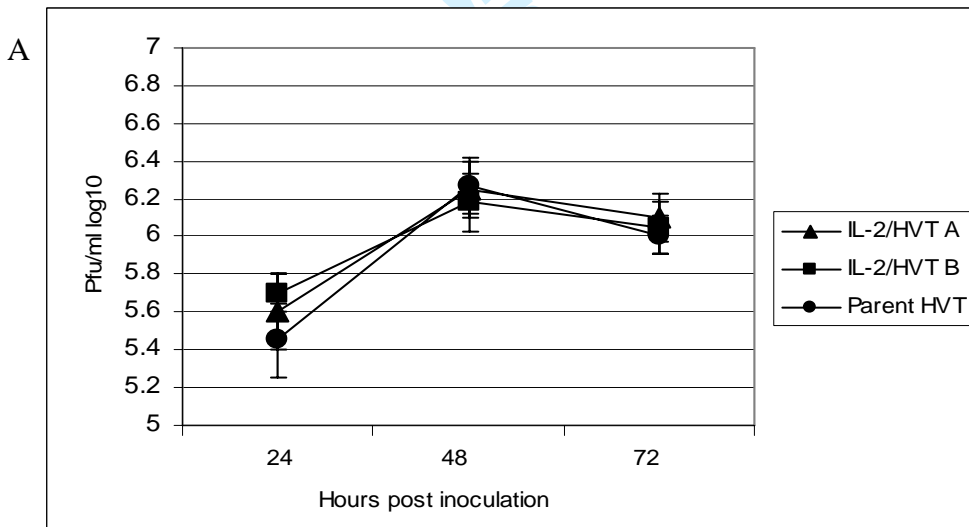
Figure 1: Characterisation of IL-2/HVT recombinants.

A) Growth of viruses in-vitro. Viruses were inoculated into primary CEFs and harvested for assays at the time points shown. Titres are the mean of two roller bottles assayed in triplicate.

B) Expression of IL-2 in-vitro. CEFs were infected at an MOI of 0.01 and supernatants harvested after 48 hours. Re-isolated viruses were cultured in CEFs for two days, the supernatant harvested and subjected to an IL-2 bioassay. Supernatants taken from infected cells were added to conA-treated chicken splenocytes for 24 hours and pulsed with 3H-thymidine for 6 hours. Each bar represents the mean counts per minute (cpm) of three replicate samples.

C) Virus re-isolation following *in-ovo* vaccination. Groups of 10 embryos were vaccinated via the amniotic route with 1000pfu. Viruses were re-isolated from peripheral blood mononuclear cells and the mean virus titres re-isolated are shown.

D) Expression of IL-2 following virus re-isolation. Re-isolated viruses were cultured in CEFs for two days, the supernatant harvested and subjected to an IL-2 bioassay. Supernatants taken from infected cells were added to conA-treated chicken splenocytes for 24 hours and pulsed with 3H-thymidine for 6 hours. Each bar represents the mean counts per minute (cpm) of three replicate samples.



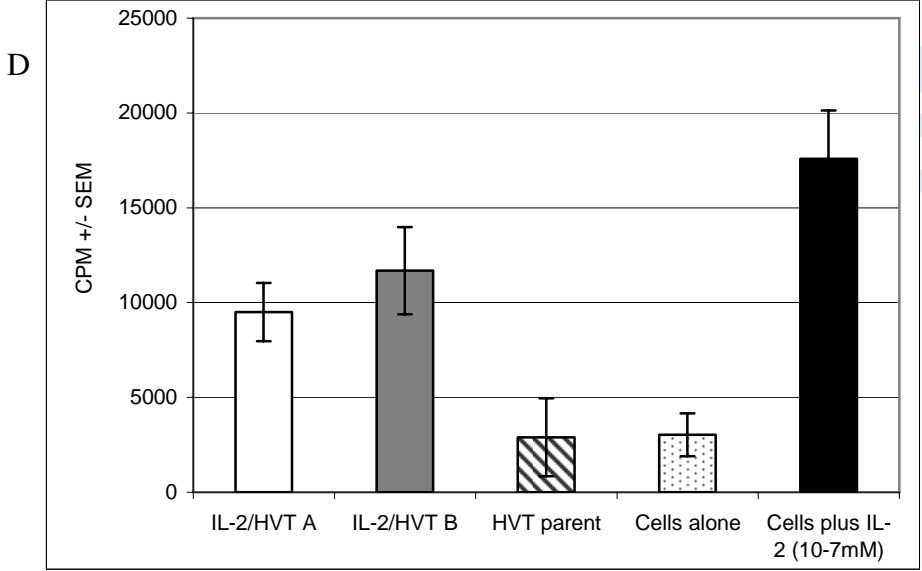
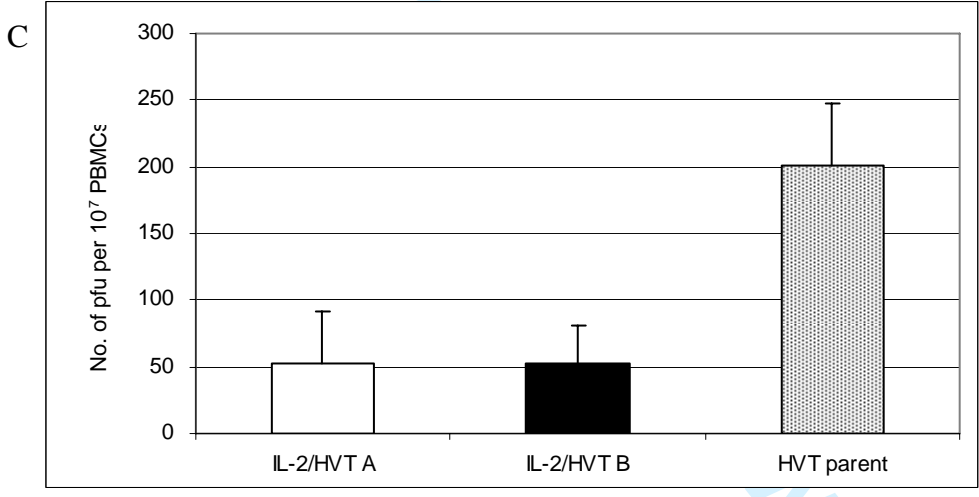
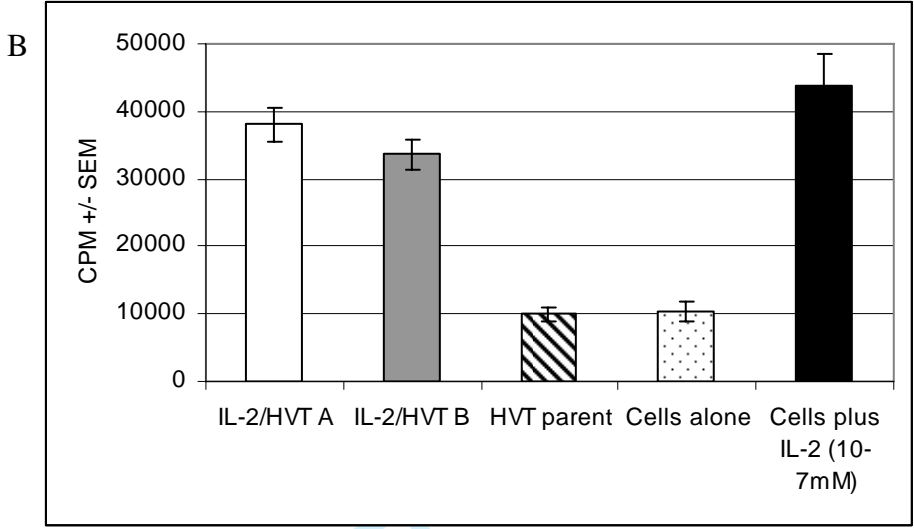
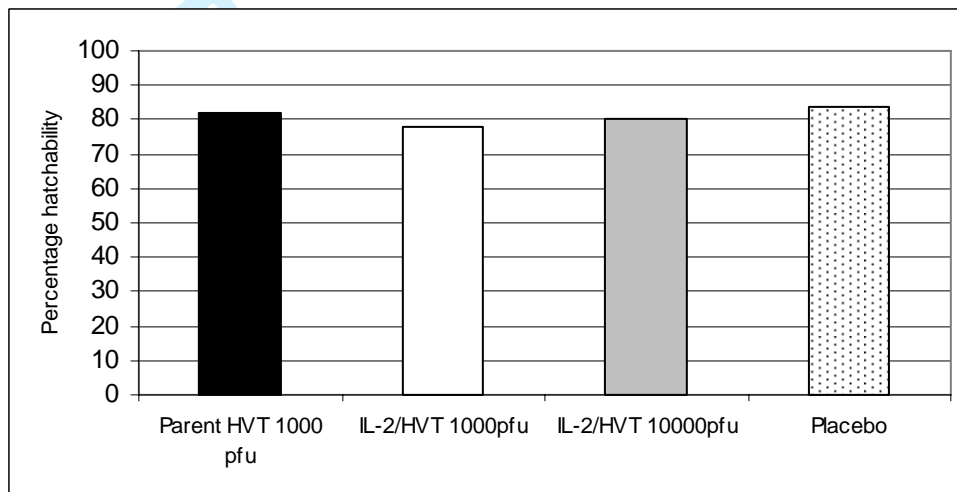


Figure 2) Safety of an overdose of IL-2/HVT *in-ovo*.

Embryonated eggs (n=100) at 18 days of incubation were inoculated with IL-2/HVT or the parent HVT in 0.1ml at the stated doses into the amniotic fluid. Hatchability was determined on day 21 of incubation and compared to placebo-vaccinated controls.



**Figure 3:** Assessment of weight gain of IL-2/HVT vaccinated chickens.

Groups of 100 18 day old embryos were inoculated via the amniotic fluid with 1000pfu of IL-2/HVT or parent HVT. Chicks were weighed at hatch and at weekly intervals for 6 weeks. Results are presented as mean weights (+/- standard deviation) based upon vaccination status and sex as shown in the legend.

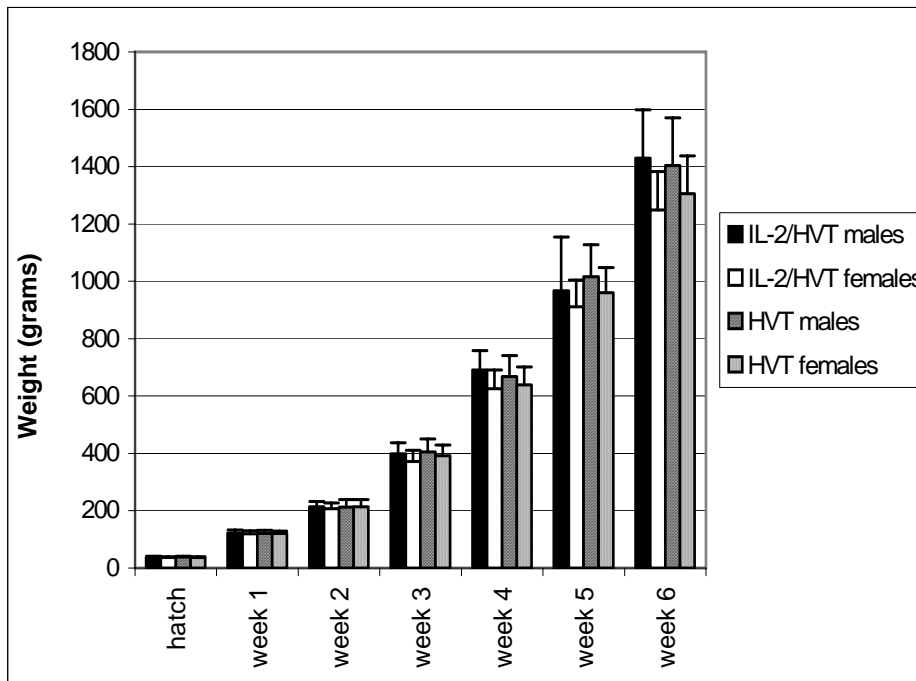


Figure 4. HVT-specific antibody response induced by IL-2/HVT.

Groups of 20 embryos were vaccinated with 1000pfu of either IL-2/HVT or HVT and allowed to hatch. The birds were bled at 3, 5 and 7 weeks of age and the sera subjected to a HVT neutralisation test. Titres are presented as the mean reciprocal dilution that neutralised 75% of HVT plaques +/- the standard deviation. Bars with superscripts indicate a significant difference in the response ( $p=0.02$ )

