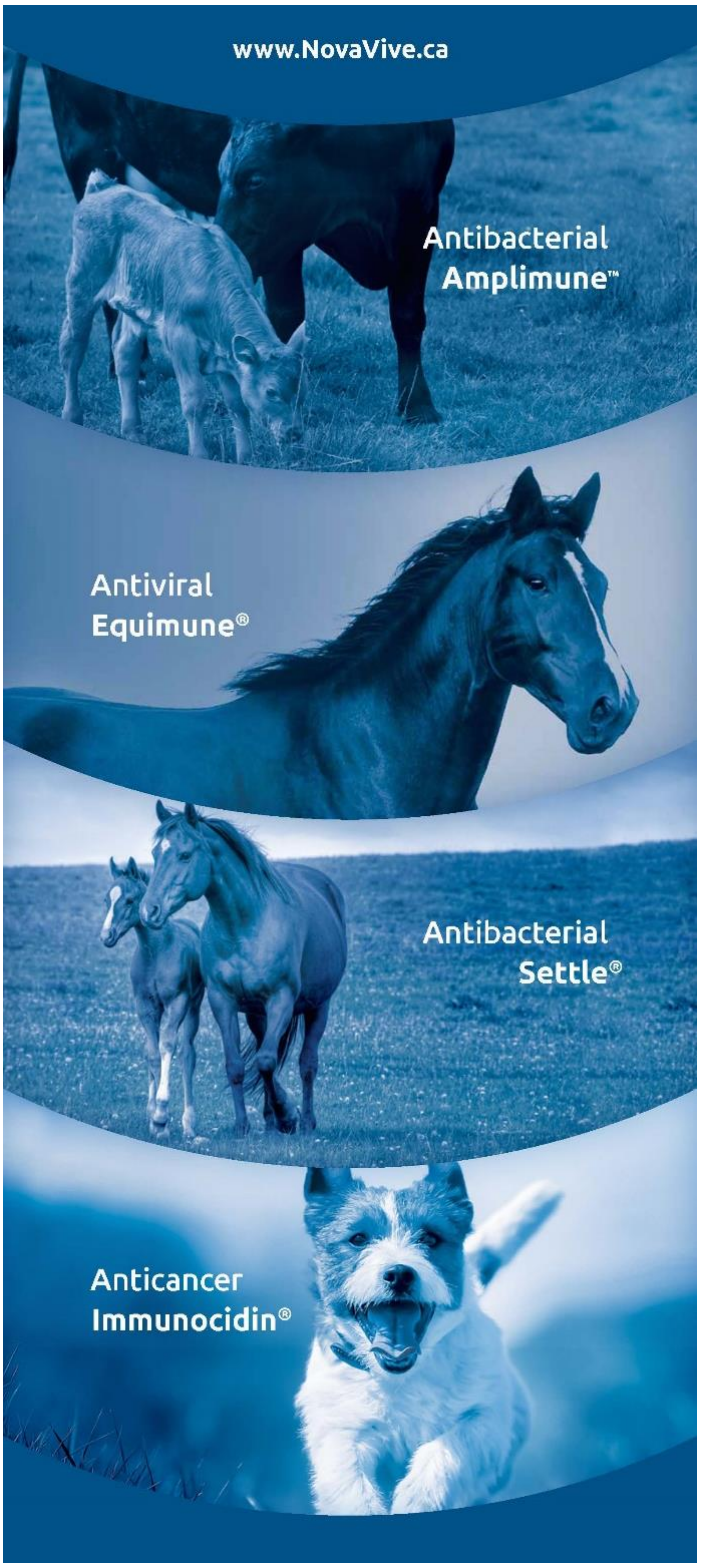
NovaVive
An immunobiology company




# Understanding the Role of a *Mycobacterium phlei* Immunostimulant in Veterinary Medicine

# CONTENTS

NovaVive Inc. (NovaVive) acquired a mycobacterium cell wall fraction (MCWF) technology in December 2014. This technology was developed by Bioniche Life Sciences Inc. (Bioniche) for a range of veterinary products; the treatment of cancer in animals and as an immunotherapeutic to treat viral and bacterial diseases.

Bioniche, now Telesta Therapeutics Inc., has further developed this technology for the treatment of bladder cancer (MCNA) in humans. NovaVive acquired the veterinary rights to the technology platform after Bioniche sold its Animal Health division to Vétoquinol S A of France in April 2014.

## About the Mycobacterium Cell Wall Fraction Technology

Mycobacterium species have been shown to be profound stimulants of the immune system. Many researchers have shown mycobacteria to be capable of fighting infections and malignancies (Zbar, 1979; Yarkoni and Rapp, 1980; Yarkoni *et al*., 1982; Mallick *et al*., 1985; Chin *et al*., 1996; Cleveland *et al*., 1996; Filion *et al*., 1999; Reader *et al*., 2001; Young *et al*., 2004). In addition, studies with other microorganisms have shown an ability to stimulate the host immune system, leading to inhibition of cancer progression. Examples include:

- the BCG vaccine strain *Mycobacterium bovis*, used in the treatment of superficial bladder cancer (Alexandroff *et al*., 1999), or
- attenuated *Salmonella* Typhimurium (Pawelek *et al*., 1997; Tome *et al*., 2013) used as a targeted anti-cancer vector, and
- anaerobic bacteria of the *Clostridium* genus (Dang *et al*., 2001; Nair *et al*., 2014) proven to enhance tumor regression in mice, and
- coryneform organisms such as *Rhodococcus equi* (Alkemade *et al.,* 1998), *Propionebacterium acnes* (Ko *et al.,* 1981), and *Corynebacterium parvum* (Halpern *et al.,* 1966; Mathé *et al.,* 1973).

One drawback is that the majority of the organisms demonstrating this ability are pathogenic. It has also been identified that specific microbial infections or the use of vaccines or therapies that mimic or, to a certain extent, repeat the immune response evoked by the intrusion of a pathogen, can elicit activation of macrophages and lymphocytes. This activation leads to the production of cytokines; especially interleukin 1 beta (IL-1β), IL-12, interferon gamma (IFNγ) and tumor necrosis factor alpha (TNF-α). These cytokines are cytotoxic agents that possess anticancer and antibacterial activity (Johnson, 1991; Neville & Pezzella, 1994; Gee *et al*., 2009; Engel & Neurath, 2010; Patyar *et al*., 2010).

The selection and enhancement of a nonpathogenic saprophytic mycobacterium (*Mycobacterium phlei*), together with proprietary growth and extraction techniques used by NovaVive, have produced a line of products (Amplimune™, Equimune®, Immunocidin®, Settle®) that have successfully avoided many of the side effects associated with other mycobacterial derived products [Freund's complete adjuvant (Goubau *et al*., 1989), *Bacillus*

*C*almette-*G*uerin (BCG) vaccine (Lamm *et al*., 1992; Curtis and Soloway, 1998; Barza *et al*., 1998; Kim *et al*., 2000)]. One aspect of the MCWF formulations, now marketed by NovaVive, is the excellent safety profile in all species and by all routes of administration.

**Immune System Response**

Application of NovaVive's MCWF immunotherapeutic technology has been shown to trigger a rapid and broad response to infection by stimulation of the innate and adaptive immunity. MCWF contains purified fragments that consist of high concentrations of muramyl dipeptides (MDP), trehalose dimycolate (TDM); lipid mycolic acid (MA); and glycolipid lipoarabinomannan (LAM), among others. These compounds have been found to display antitumoral and immunostimulating activities (Chedid *et al.,* 1972; Bernstein *et al.,* 1991; Filion *et al*., 1999; Filion *et al*., 2000; Morales *et al*., 2001). It has been demonstrated that *Mycobacterium phlei* DNA (in the form of short oligonucleotides) when associated with the bacterial cell wall, inhibited the proliferation of bladder cancer cells by inducing apoptosis (Morales *et al.,* 1995; Filion and Phillips*,* 1997; Filion *et al.,* 1999).

- Lipid mycolic acid (MA) has been found to stimulate transitory neutrophils, as well as provoke the production of IL-12, IFNγ, IL-6, and myeloperoxidase while suppressing IL-10 production and enhancing TNFα production (Korf *et al.,* 2005). IL-12 (sometimes referred to, as the anticancer cytokine) has been related to the antiangiogenic activity associated with MCWF (Voest *et al*., 1995). MCWF was able to stimulate levels of IL-12 equivalent to lipopolysaccharide (Filion *et al.,* 2000).
- Trehalose dimycolate (TDM), or cord factor, is a known inducer of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα (Indrigo *et al.,* 2003).
- Muramyl dipeptides (MDP), the smallest peptidoglycan component of MCWF, up-regulates the production of IL-2, IL-4, IL-10, IL-12 and IFNγ (Filion *et al.,* 1999; Filion *et al.,* 2000).
- Glycolipid lipoarabinomannan (LAM) has been identified as a trigger for the production of TNF-α, GM-CSF, TGFβ, IL-1α, IL-β, IL-6, IL-8, IL-10, and IL-12, as well as being a chemoattractant for monocytes and neutrophils (Strohmeier and Fenton, 1999).

MCWF has been demonstrated to interact with common signalling pathways used by different cell types of the immune system.

*Mycobacterium phlei* DNA associated with the bacterial cell wall (cell wall complex), as well as purified *M. phlei* DNA, was shown to be a potent inducer of IL-6, IL-8, IL-12, IL-18 and TNF-α synthesis by monocytes and macrophages *in vitro* and *in vivo* (Filion *et al*., 1999; Filion *et al.,* 2000), and was capable of inhibiting tumor cell division and inducing apoptosis in cancer cells (Filion *et al*., 1999; Kabbaj and Phillips, 2001; Reader *et al*., 2001).

MCWF activates macrophages and lymphocytes inducing and enhancing both cell mediated and humoral immune responses.

In addition, it appears that many distinct mycobacterial cell wall components may interact with different members of the Toll-like receptor (TLR) family. It has been shown that LAM is recognized through TLR-4, while phosphatidylinositol mannosides (PIMs) serves as a ligand for TLR-2 (Means *et al.,* 2003; and Velasco-Velazquez *et al.,* 2003). Thus, MCWF technology is able to increase the likelihood that an invading pathogen will be recognized by more than one of the host's immunological mechanisms.

The NovaVive MCWF products were the first immunotherapeutic technologies approved by the United States Department of Agriculture (USDA) for use in animals. The species *M. phlei* has been the preferred organism in the production of biological products by NovaVive due to the fact that this soil-borne organism does not carry the *Mycobacterium bovis* (bTB) or *Mycobacterium avium* subsp. *paratuberculosis* (MAP) antigens, and therefore does not elicit a positive serological bTB or MAP reaction (Data on file, Study ID # BIS-40). As a result, the NovaVive MCWF technology can be used in food producing animals with no risk of eliciting a false-positive reaction.

MCWF has been demonstrated to interact with common signalling pathways used by different cell types of the immune system. MCWF activates macrophages and lymphocytes inducing and enhancing both cell mediated and humoral immune responses (Alkemade, 1989; Wade and Alkemade, 1997; Filion *et al*., 1999; Filion *et al*., 2000; Morales *et al*., 2001).

MCWF exerts additive or synergistic effects with innate and adaptive immunity mechanisms due to its influence on the production of proinflammatory cytokines (such as IFNs, IL-1β and TNF-α) (data on file, Study ID # Regressin, MCW Research Summary). Innate immune mechanisms are used by the host to respond to pathogens. After detection, a battery of antimicrobial mechanisms is deployed to kill bacteria in infected cells. In response to inflammatory stimuli generated by pathogens, macrophages and neutrophils produce chemically reactive molecules such as antimicrobial peptides and a rapid burst of reactive oxygen species (ROS) followed by prolonged production of reactive nitrogen intermediates (RNI) (Radtke and O'Riordan, 2006). Nitric oxide (NO) and RNI are generated by nitric oxide synthases (NOS) (MacMicking *et al.,* 1997). In macrophages, inducible NOS (iNOS) is important for the generation of RNI during inflammation, immune regulation (MacMicking *et al.,* 1997; Bogdan, 2001; Lowenstein and Padalko, 2004) and infection with intracellular pathogens (Chakravortty and Hensel, 2003; Fang, 2004). Nitric oxide (produced by iNOS), mediates tumoricidal and bactericidal actions throughout the body. The gene encoding iNOS is not transcribed in uninfected cells, but its expression can be induced by proinflammatory cytokines and lipoteichoic acid (Chakravortty and Hensel, 2003; Fang, 2004). Stimulation results in the activation of signalling pathways leading to iNOS transcription (Fang, 2004).

In this paper, we review the available clinical data from all four commercial MCWF products, discussing their use in the prevention and treatment of infectious diseases in the bovine and equine species and the treatment of malignancies in equine and canine species.

## AMPLIMUNE™

An immunostimulant approved for the treatment of K99 *Escherichia coli* scours in calves.

Amplimune was previously sold in the U.S.A. as Immunoboost®.

Studies have shown that 1 mL of Amplimune administered to the calf after 24 hours of age or at the first sign of infection and a second dose 7 days later is effective.

Sub-cutaneous and intramuscular administration have been shown to be as effective as I.V.

**Amplimune** activates an innate immune response to recognize, react to, and recover from, infections. It is recommended that Amplimune be administered intravenously to calves one to five days of age, but it has been administered by veterinarians and in research studies: at various ages and weights, using different administration routes; safely and effectively (Nosky and Worthington, 1996; Nash, 2003). Amplimune has been shown to be effective even under stress situations when cortisol levels are increased (Griebel, 1999).

### K99 *E. coli* scours

Amplimune was evaluated as a single treatment in calves challenged with *E. coli* K99. Twenty-two Holstein bull calves, within two hours of birth, were randomly assigned to a treatment or control group. Calves were colostrum-deprived and only fed milk replacer that did not contain antibiotics, every eight hours throughout the study. Calves were challenged orally (at approximately six hours of age) with an average of 3.8 $x10^9$ cfu (range 2.8 $x10^9$ to 5.6$x10^9$) of pathogenic *E. coli* K99. Calves in the treatment group received one dose of Amplimune intravenously at the first signs of infection. No other treatment or supportive therapy was administered. Calves in the control group did not receive any treatment. Results of this study demonstrated that calves treated with Amplimune had a significantly higher survival rate as compared to the control group (90% versus 42%). Surviving calves treated with Amplimune demonstrated higher weight gains compared to surviving control calves. At the end of the study, Amplimune-treated calves sold for more than twice the price of control calves (Data on file, Study ID# BEC-13).

Nosky and Worthington conducted a controlled study in 400 hutch-reared calves (colostrum status unknown), in which they evaluated health outcomes and economic advantages resulting from the administration of Amplimune. The study demonstrated the therapeutic potential of MCWF to protect against various organisms including *E. coli* K99. It was demonstrated that when Amplimune was administered to calves at less than 24 hours of age, there was a significant positive effect on weight gain, health and production economics; including a reduction in medication cost. The study also evaluated the effects of intravenous (IV), intramuscular (IM) and subcutaneous (SC) administration routes on the overall performance of these calves. It was observed that the SC route of administration showed advantages over IM and IV administration (Nosky and Worthington, 1996).

**Effect on bovine blood leukocyte populations**

The effect of Amplimune on the immune system of neonatal calves was evaluated in a study performed at the Veterinary Infectious Disease Organization (VIDO) research facility (University of Saskatchewan, Saskatoon). This study evaluated the ability of Amplimune to alter blood leukocyte populations. Fifteen newborn colostrum-deprived male Holstein calves were randomly allocated to 3 study groups. A control group received a SC injection of physiological saline; one Amplimune (Immunoboost)-treated group received a single IV injection of 1 mL; and a second Amplimune (Immunoboost)-treated group received a single SC injection of 1 mL. During the first days of life, it was observed that cortisolemia had an influence on the low level of immune system activation. But in calves from the SC Amplimune (Immunoboost)-treated group, the number of activated T-lymphocytes (MHC Class II CD4 cells) increased on day four post-treatment, suggesting that a SC injection of Amplimune (Immunoboost) may be able to overcome some of the immune suppression induced by endogenous cortisol (Griebel, 2003).

Amplimune is a USDA-approved biological therapy (derived from a non-pathogenic bacterium) that stimulates a calf's immune system to establish an early, normal immune response to help overcome neonatal diarrhea caused by K99 *E. coli.*

**Effect on health and performance parameters**

Studies demonstrate that Amplimune will improve weight gain and overall health, decrease antibiotic usage, and increase profits in commercial calf operations. If the immune function can be optimized early in a calf's life, it is more likely to remain healthy and maximize its production potential. More energy can be directed to growth when it is not required for disease fighting activity.

David Nash in 2003 evaluated the effects of Amplimune (Immunoboost) on health and performance of 3,965 light Holstein steer calves, weight 84 – 127 kg (185 – 280 lb) in a commercial feedlot situation. In half of the animals, Amplimune was administered SC as a prophylactic treatment. It was concluded that the use of Amplimune showed a positive effects on the measured health and performance parameters. Amplimune used as a prophylactic treatment resulted in decreases in mortality, cull rate and total treatment costs, netting a positive 20.8% return on investment for the Amplimune (Immunoboost)-treated calf group in this study (Nash, 2003). These results are consistent with observations made from previous studies (Nosky and Worthington, 1996).

## EQUIMUNE®

An immunostimulant that is approved by the USDA for intravenous administration to horses for the treatment of Equine Respiratory Disease Complex.

Eighty-five percent (85%) of horses treated with Equimune returned to normal within seven days. In contrast, only 35% of those treated with placebo and conventional therapies recovered within the same period of time.

**Equimune** is a potent stimulator of mononuclear cells, inducing the release of IL-1. Clinical studies have shown that horses treated with Equimune recover from respiratory disease complex sooner than horses treated with antibiotics alone. In the early stages of a viral infection, Equimune reduces the time available for viral replication, the number of tissue cells affected, the degree of cellular damage and the duration of clinical signs and physiological abnormalities.

**Equine Respiratory Disease Complex (ERDC)**

Canadian equine practitioners from the province of Ontario participated in an Equimune efficacy study for the treatment of equine upper respiratory disease (Fall/Winter of 1985-1986). The horses treated in this study were all Standardbreds except for one which was an American Quarter Horse cross. The age of the horses ranged from 6 months to 6 years with an average age of 2.32 years. This study demonstrated that one intravenous (IV) injection of Equimune was effective for the treatment of equine upper respiratory disease of tentatively diagnosed viral origin. Once confirmed by positive seroconversion, the infectious agents included equine herpes virus type 1, Influenza A equine-1 (H7N7) and Influenza A equine-2 (H3N8) (Data on file, Study ID # E.I.S.-2).

From November 1985 to April 1986, a survey form was provided with each syringe of Equimune purchased. Data was collected from 27 veterinarians who used Equimune in their practice during this period. The data provided enabled the tabulation on Equimune efficacy and safety based on the clinical evaluation of practitioners. After the statistical evaluation of 104 clinical reports, it was concluded that only one IV injection of Equimune was required for the treatment of equine upper respiratory disease (Data on file, Study ID # E.I.S.-3).

The efficacy and safety of Equimune was tested in a randomized, double-blinded study. Fifty-three Standard bred horses diagnosed with ERDC were included in this study. It was unknown to the attending veterinarian whether each individual syringe contained Equimune or placebo. Only one injection was given in the course of the disease. During follow-up examinations, the veterinarians could prescribe additional conventional therapy if they felt it was indicated. Seventy-three percent (73%) of the horses which received a single dose of Equimune required no concurrent therapy. Eighty-five percent (85%) of horses treated with Equimune returned to normal within seven days.

In contrast, only 35% of those treated with placebo and conventional therapies recovered within the same period of time (Cormack *et al*., 1991).

**Vaccine Adjunct**

Another Equimune application is as a vaccine adjunct. For example, Equimune was used in conjunction with a commercial West Nile virus (WNV) vaccine in horses. WNV is a mosquito-borne zoonotic arbovirus that has rapidly spread throughout North America. This virus has been identified in all of the continental United States (US), most of Canada and Mexico. WNV infection causes inflammation of the brain and spinal cord of horses, producing neurological symptoms. Since 1999, over 25,000 cases of WNV encephalitis have been reported in US horses. Horses represent 96.9% of all reported non-human mammalian cases of WNV disease (Ostlund *et al*., 2001). The case fatality rate for horses exhibiting clinical signs of WNV infection is approximately 33%. Thus, vaccination for West Nile virus is highly recommended and is considered an essential standard of care for all horses in North America. Although the vaccine is reported to be 95% effective in challenge studies, measurable antibody production (seroconversion) does not occur in all animals. This lack of seroconversion may signify less than optimal protection (Cortese *et al*., 2013).

A 50-horse study was designed to investigate the ability of the immunostimulant, Equimune, to increase the number of horses that would seroconvert following vaccination. The study involved the concurrent administration of a single dose of Equimune together with the sensitizing West Nile vaccination at day 0 (group 1); Equimune at both the sensitizing (day 0) and booster (day 21) dose (group 2); a half-dose of Equimune together with the sensitizing West Nile vaccination at day 0 (group 3); a half-dose of Equimune at both the sensitizing (day 0) and booster (day 21) dose (group 4); and a positive control group that received only West Nile vaccine on day 0 and a booster on day 21 (group 5). Results support the following conclusions regarding the use of Equimune as a concurrent adjunct to WNV vaccination. A full-dose of Equimune triggered more animals to seroconvert (78% and 80% compared to 56% for controls at day 45). A full-dose of Equimune triggered earlier seroconversion (50% and 78% compared to 44% for controls, at day 30). Ten percent (10%) of all horses receiving Equimune (4 of 39) developed measurable seroconversion within 21 days following the sensitizing vaccine dose. None of the positive controls showed seroconversion at day 21. A less than optimal dose of the immunostimulant does not appear to be as effective as the full-dose (70% and 60% seroconversion for half-dose regimens compared to approximately 80% for full-dose regimens) (Data on file, Study ID # Equimune potentiation of a commercial West Nile virus vaccine in horses).

## IMMUNOCIDIN®

An immunostimulant that is approved for treatment of mixed mammary tumors and mammary adenocarcinomas in dogs and equine sarcoids in horses.

Preliminary research indicates that Immunocidin has great potential in the treatment of different canine cancer types, including:

- osteosarcoma,
- transmissible venereal tumors,
- appendicular osteosarcoma
- muscle-invasive transitional cell carcinoma,
- salivary gland carcinoma, and
- lymphoma.

**Immunocidin** stimulates the activation of neutrophils, macrophages, and lymphocytes which enable tumour suppression and reduced growth. Immunocidin is administered via intratumoral (IT) injection, but the response to this immunotherapeutic agent is generalized, and untreated sites frequently undergo regression.

## CANINE

### Mixed mammary tumor & mammary adenocarcinoma

MCWF efficacy against mixed mammary tumors and mammary adenocarcinomas in dogs has been evaluated in veterinary practice since 1983. Efficacy data for the use of Immunocidin for the immunotherapy of mammary tumors in dogs has shown that a high percentage of dogs experience more than 50% regression after IT injections with Immunocidin (Data on file, Study ID # VR-C-LIC-I-1989).

Two studies were conducted to evaluate the safety of IV administration of Immunocidin in healthy dogs. In these studies, there were no clinically significant adverse events observed. In addition, no macroscopic or microscopic changes were observed in any of the examined organs (lungs, liver, spleen, and bone marrow). These safety studies included 9 healthy dogs divided into two experimental groups that included a lower and a higher Immunocidin dosage. Immunocidin was administered intravenously to all dogs. In one study design, all dogs received 4 Immunocidin injections every two weeks. These dogs were euthanized and subjected to necropsies two weeks following the last Immunocidin injection. In the other study design, all dogs received 3 Immunocidin injections every week and 2 dogs were euthanized and subjected to necropsies 7 days after the last Immunocidin injection. The rest of the dogs were euthanized and evaluated 4 weeks following the last injection (Mangieri *et al*., 2015).

| Canine *Mammary Adenocarcinoma* - Data Summary | | | | | | | |
|---|---|---|---|---|---|---|---|
| Breed | Age (Years) | Sex | Location | Initial Size ($cm^3$) | Times Treated | Total Dose (mL) | Results |
| Mixed | 7.5 | F | R-3 | 93.8 | 3 | 20.0 | 80% regression (surgically excised) |
| Mixed | 10 | F (spayed) | R-5 | 0.8 | 2 | 3.5 | Remission |
| Dachshund | 8 | F | L-3 | 218.0 | 6 | 30.0 | Remission |
| Terrier/ Chihuahua | 11 | F (spayed) | R-5 | 2.3 | 3 | 6.0 | Remission |
| Sheltie | 14 | F | R-5 | 2.4 | 6 | 9.0 | Remission |
| Dachshund | 8 | F | L-4 | 4.0 | 3 | 4.5 | Remission |
| **Canine *Mixed Mammary Tumor* - Data Summary** | | | | | | | |
| Breed | Age (Years) | Sex | Location | Initial Size ($cm^3$) | Times Treated | Total Dose (mL) | Results |
| Poodle | 11 | F | R-4<br>L-3 | 0.5<br>9.6 | 3<br>3 | 3.0<br>9.0 | Remission |
| Poodle | 10 | F | R-4<br>L-3 | 4.0<br>16.2 | 2<br>2 | 4.0<br>10.0 | Non-related death<br>58-60% remission |
| Poodle | 10 | F | R-3<br>R-4<br>L-4 | 5.9<br>1.2<br>2.2 | 3<br>3<br>3 | 3.5<br>2.25<br>2.0 | Remission<br>Remission<br>Remission |
| Boston Terrier | 13 | F (spayed) | R-2 | 1.7 | 3 | 3.5 | Remission |
| Mixed | 14 | F | R-3 | 21.9 | 3 | 6.0 | Remission |

## Osteosarcoma

Eighteen dogs diagnosed with osteosarcoma (OS) were treated with Immunocidin in conjunction with appendicular amputation. Immunocidin was administered intramuscularly (IM) on the day of surgery and once a week following surgery for the first month, then every two weeks for the next three months. This study demonstrated that Immunocidin treatment following surgery has a beneficial effect in dogs diagnosed with OS. The survival rate was 50% at 36 months post-diagnosis in localized OS patients (N=12) and 50% at 12 months in patients with locally invasive OS and/or regional lymph node involvement (N=6) (Data on file, Study ID # Efficacy of Mycobacterial Cell Wall Extract (MCWE) in the treatment of osteosarcoma in dogs).

In another study, eight dogs diagnosed with osteosarcoma (OS) were treated with Immunocidin in conjunction with the alkylating agent Cisplatin, and appendicular amputation. Forty-eight hours after amputation, patients received the following treatment: Six intravenous (IV) doses of Cisplatin (70 mg/m2, every 4 weeks, slow drip method). Immunocidin was administered IV every week during the first 4 weeks (single-bolus method), and in conjunction with Cisplatin afterwards, until the endo of the treatment. Results showed a survival rate of 87.5% after 6 months and a disease-free survival rate of 75%, twelve months after surgery (Mangieri *et al.,* 1995).

**Transmissible Venereal Tumors**

The effect of Immunocidin as an adjunct to chemotherapy in the treatment of transmissible venereal tumors (TVT) has been evaluated. In one study, 17 dogs with TVT confirmed by cytology were included in this study. Vincristine was administered two times, seven days apart and MCWF was administered by IV injection once a week for four weeks. The first two Immunocidin administrations were concurrent with the chemotherapy, while the last two were administered as a single anti-cancer agent. Immunocidin had a beneficial effect and significantly reduced the use of vincristine and increased the complete response rate (100% complete response 29 days post-initial treatment) (Mangieri, 2003).


**(A) Left: TVT immediately after 1st Immunocidin administration. Right: TVT after 3rd Immunocidin administration.**

**(B) Left: TVT immediately after 1st Immunocidin administration. Right: TVT after 4th Immunocidin administration.**

Eighty-four (84) dogs histopathologically confirmed (including lymphadenopathies) were involved, in a comparative study, were non-surgical treatments for TVT were evaluated. The dogs were of varying age (between 1.5 and 10 years), 36 males and 48 females. The three most represented breed were: mixed breed (n = 50), German shepherd (n = 18) and Dobermann (n = 7); the remaining were, Cocker Spaniel, Pointer, Terrier, Dachshund or Bobtail. Differences across breed were not considered to be a factor in the level of response to the treatments.

The different treatments were:

I. Vincristine (0.5 mg/m$^2$, IV once a week) (11 dogs)
II. Vincristine (0.8 mg/m$^2$, IV once a week) (46 dogs)
III. Vincristine (0.5 mg/m$^2$, IV once a week) plus cyclophosphamide plus methotrexate (11 dogs)
IV. Vincristine (0.8 mg/m$^2$, IV once a week) plus intratumoral (IT) injection of mycobacterium cell wall fraction (MCWF) (10 dogs)
V. MCWF (IT, once a week) (6 dogs)

Results showed that the treatments II and IV achieved better outcomes. The presence of lymphadenopathies was more prevalent in males (36.1% vs 2.1%), and only treatments II, III and IV were successful in treating these lymphadenopathies. From this study, the recommended treatment was: Vincristine (0.8 mg/m$^2$, IV once a week) plus IT injection of MCWF, weekly for 2 or 3 doses. In tumors that are 3 cm or less in size, the recommended treatment is 3 doses of MCWF (IT), once a week (Mangieri J., *et al*. 1995).

Mangieri and collaborators have concluded that chemotherapy plus immunotherapy have surpassed the surgical treatment of canine TVT. The addition of MCWF (IV) to chemotherapy enables the patients to tolerate the adverse effects caused by chemotherapeutic agents; in addition, the decreased production of neutrophils (neutropenia) caused by chemotherapy was not observed in dogs where MCWF was administered.

**Appendicular Osteosarcoma**

The effect of Immunocidin after standard chemotherapeutic treatment of 8 dogs diagnosed with appendicular OS was also evaluated. Standard treatment included surgical removal (amputation) of the affected limb and IV administration of cisplatin. Cisplatin was administered 6 times at three-week intervals starting from the day of surgery. Immunocidin was administered by IV injection once a week for twelve weeks starting after the last cisplatin treatment. This study demonstrated that Immunocidin, as an adjunct to standard therapy in the treatment of appendicular OS, has the potential to increase the median survival rate in canine oncology patients from the current approximately 12 months to more than 18 months (50% survival rate at 18 months) (Mangieri *et al*., 2003).

**Transitional Cell Carcinoma**

Five dogs with transitional cell carcinoma of the lower urinary tract were solely treated with Immunocidin administered intravesically. These dogs presented with tumors that were recurrent, surgically unresectable, failed chemotherapy, or their owners had declined standard care of treatment. A dose of Immunocidin was administered weekly for 4 treatments (3 dogs), and during 3 consecutive days (2 dogs), followed by treatment every 14 or 28 days depending on tumor and clinical response. No significant toxicity was documented, and significant improvement in quality of life was observed. Hematuria, dysuria, and pollakiuria were resolved or reduced in all treated dogs 14 days after the start of treatment. Tumor size and disease progress was monitored and measured monthly by ultrasound. Preliminary data showed that 3 dogs achieved stable disease status and 2 dogs achieved partial remission. Three dogs have continued to receive regular Immunocidin immunotherapy over the last 18, 15 and 3 months (Rodrigues *et al*., 2015).

**Salivary Gland Carcinoma**

NovaVive has obtained comprehensive data on the remission of recurrent salivary gland carcinoma in a dog following Immunocidin treatment. A five year old Collie was diagnosed with poorly differentiated salivary gland carcinoma. Previous treatments included three surgical interventions in combination with chemotherapy. After the third tumor recurrence, Immunocidin was introduced as a stand-alone treatment. Immunocidin was administered IT on a weekly basis. After only two IT injections of Immunocidin, a 10% tumor size reduction and the stabilization of the disease were observed. Two additional administrations of Immunocidin contributed to tumor partial response (PR) (characterized with progressive tumor ulceration and flattering). Six additional Immunocidin treatments were performed until complete remission was observed (Data on file, Study ID # NV-C-SGS-I-2015).

**Lymphoma**

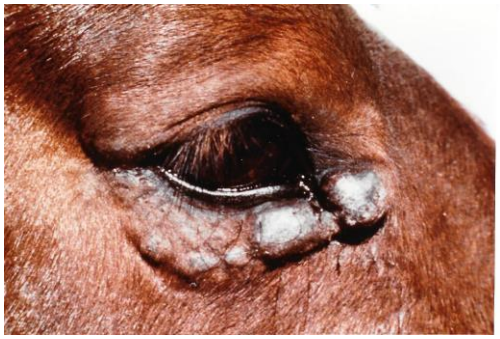
A dog with histologically confirmed multicentric lymphoma without evidence of metastasis and enlarged lymph nodes was placed on Immunocidin therapy. Treatment regimen included weekly administration of Immunocidin IV (slow drip method). Up to date, a total of 12 Immunocidin treatments were performed and dog survival time is at five months (2 more than the average median survival rate for standard lymphoma protocols that include chemotherapy). There are no signs of metastasis, lymph nodes are of normal size and significant improvement in quality of life is observed. This patient continues to receive Immunocidin weekly (Data on file, Study ID # NV-C-L-I-2015).

# EQUINE

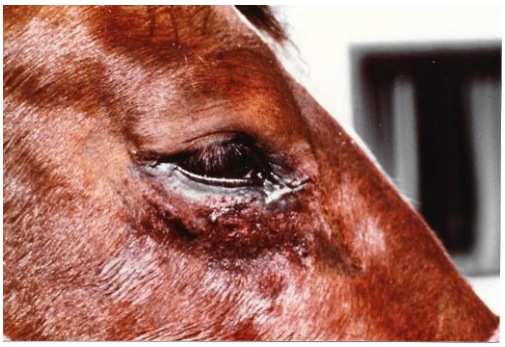
## Equine Sarcoids

Equine sarcoids are locally aggressive fibroblastic neoplasms considered to be the most common skin tumors of horses worldwide. Equine sarcoids have been linked to infection of the horse with bovine papillomavirus (BPV) types 1, 2 and 13 (Lancaster *et al.,* 1977; Sundberg *et al*., 1977; Otten *et al.,* 1993; Lunardi *et al.,* 2013). The fact that viral DNA was only found in sarcoid tissues in most studies infers a relationship between viral infection and neoplastic transformation. Equine sarcoids have been shown to be responsive to immunotherapy with Immunocidin (Murphy *et al.,* 1979). A study using Immunocidin for the treatment of equine sarcoids demonstrated that 73% of the cases underwent complete regression and 27% of the cases experienced partial regression (Data on file, Study ID# ER-1). Immunocidin possesses antiviral and antitumor capabilities, making it a successful equine sarcoid treatment. Immunocidin has been successfully used by veterinarians for the treatment of facial equine sarcoids.

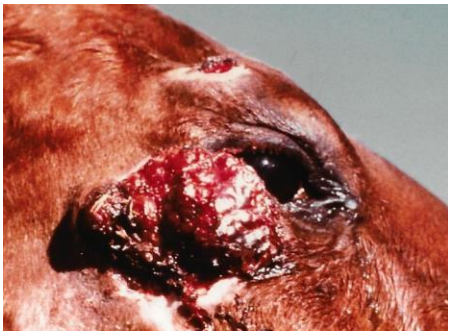
### Two cases of facial sarcoid treated with Immunocidin



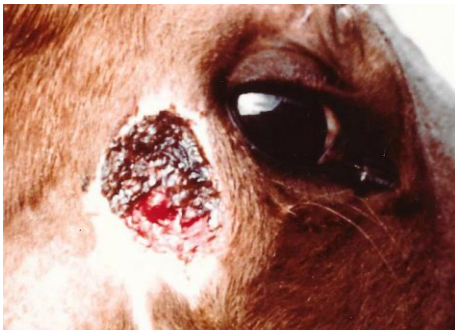
Prior to treatment



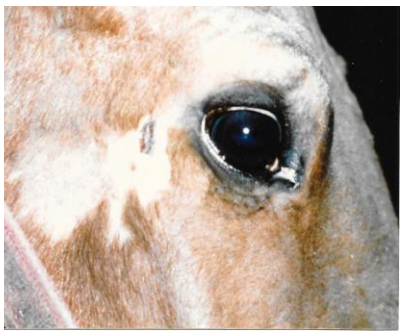
Seventeen (17) days after third Immunocidin treatment



Recurrent sarcoid above and below the eye. Upper tumour resected; Lower tumour partially resected and treated four times with Immunocidin at 7-day intervals



Seven (7) days after 3rd Immunocidin treatment



Approximately one month after 4th Immunocidin treatment.

## SETTLE®

An immunostimulant for the non-antibiotic treatment of endometritis in mares.

Settle is a safe, effective, non-antibiotic therapy for nursing mares.

Intrauterine and intravenous routes of administration are effective in clearing endometrial infection.

**Settle** is approved for the non-antibiotic treatment of endometritis in mares. Endometritis is an important disease that has high economic impact on the horse industry as it causes infertility in mares (Troedsson, 1995; Troedsson, 1997; Troedsson, 1999; Watson, 2000). In general, there are two groups of mares: Those capable of clearing bacteria that contaminate the uterus following parturition or breeding; and those that are prone to infection (Katila, 1996). Traditionally, endometritis in mares has been treated using antibiotics and oxytocin (LeBlanc, 1994). However, data indicate that non-specific stimulation of the immune system with Settle will restore homeostasis of local inflammatory mechanisms, and consequently assist in the treatment of this condition in mares (Fumuso *et al.,* 2003a).

In mares, foal heat is well-known to be associated with a poorly involuted uterus which is inflamed and grossly contaminated with microorganisms as a result of recent parturition. These factors combine to provide a low fertility rate. The potential for Settle to improve the conception rate at foal heat was evaluated; eighty-one mares were treated with either Settle (n=43) or a placebo (n= 38) at 24 hours after foaling. A representative group was sampled 1 day (S1) and 7 days (S2) after foaling for evaluation of bacterial load and exfoliative cytology scoring of polymorphonuclear neutrophils (PMNs).

| | Placebo | | Settle | |
|---|---|---|---|---|
| **Pregnancy** (n=81) | 9/38 (24%)* | | 28/43 (65%)* | |
| | S1 | S2 | S1 | S2 |
| Bacteriology (n=25) | 1-4 (3)* | 0-3 (3)* | 0-4 (3)* | 0-0.2 (0)* |
| *Isolates (n)* | | | | |
| *S. zooepidemicus* | 8 | 6 | 4 | 1 |
| *S. equisimilis* | 1 | 1 | 2 | 0 |
| *S. equi* | 1 | 0 | 0 | 0 |
| *E. coli* | 5 | 2 | 7 | 1 |
| *Klebsiella* spp. | 0 | 0 | 1 | 0 |
| *Proteus* spp. | 1 | 0 | 0 | 0 |
| Cytology (n=25) | 12.4-60 (30.1)* | 0.8-35 (14.9)* | 12.8-60 (21.8)* | 0-0.25 (0.8)* |

*Median.

Fifteen days after foaling; all mares were evaluated by ultrasonography. The control group experienced no significant change in bacterial load during the one week following foaling, while 85% of the Settle-treated

mares had achieved bacterial clearance ($p<0.0003$) and a dramatic decrease in clinical cytology scores ($p<0.001$) within one week following foaling (Fumuso *et al.,* 2003b).

Ultimately, approximately 3 times more Settle-treated mares conceived during foal heat than the control group ($p=0.0003$), clearly demonstrating that Settle is a safe, effective, non-antibiotic therapy for nursing mares.

In another study, 30 barren mares susceptible to endometritis, (as indicated by the presence of uterine fluid) during both diestrus and estrus, were selected from a herd of 896 mares. All mares were treated with antibiotics to clear the existing infection, and upon clearance, estrus was induced by an intramuscular injection of Prostaglandin-F2α. Twenty-four hours after the onset of estrus (confirmed by ultrasonography), endometritis was induced by an intrauterine inoculation of a 5 x$10^6$ cfu *Streptococcus zooepidemicus* culture. Endometritis was confirmed by exfoliative cytology, bacterial culture, uterine biopsies and ultrasonography. Following detection of endometritis, mares were randomly allocated into four experimental groups and treated as follows: a) 10 mares treated with Settle by intrauterine (IU) administration; b) 10 mares treated with Settle by IV injection; c) 5 mares treated with placebo by IU infusion; and d) 5 mares treated with placebo by IV injection. Ovulation occurred 3 to 6 days after infection. Mares were evaluated 1 day post-ovulation and again 7 days post-ovulation for uterine fluid accumulation via transrectal ultrasonography. They were also sampled for bacteriology, exfoliative cytology, and uterine biopsy to determine the degree of clearance of the previously established *S. zooepidemicus* infection. Mares treated with Settle either IV or IU experienced a statistically significant decrease ($p<0.001$) in the number of PMNs in biopsy samples at 24 hours and 7 days post-treatment as compared to pre-treatment samples. Additionally, a statistically significant decrease ($p<0.001$) in the number of bacteria was observed at both 24 hours and 7 days post-treatment; and a statistically significant reduction ($p<0.001$) in the amount of uterine fluid was observed at 7 days post-treatment. Endometritis was observed in all placebo-treated control mares after 7 days (Rogan *et al*., 2007).

Following treatment with Settle, 35% of all the treated mares had responded successfully by the time of ovulation and 70% by 7 days post-ovulation. In addition, results from this study confirm that both intrauterine and intravenous routes of administration are effective in clearing endometrial infection and no side effects were observed.

In Argentina, approximately 15% of the mares are considered sub-fertile and are known as problem breeding mares. The potential of Settle to ameliorate the breeding response of 36 cross-breed barren mares with a history of failing to conceive in the previous 2 years was evaluated. The presence and extent of endometritis was evaluated using bacteriological culture, exfoliative cytology and endometrial biopsy, both before treatment and 21 days following exposure to fertile stallions. Animals were treated during oestrus with Settle IV (n=17) or IU (n=19). While there was no difference between the routes of administration, treatment with Settle resulted in 39% of the infertile mares becoming pregnant. Fourteen percent of the treated mares failed to clear their original uterine infection; however, their biopsy results showed a statistically significant improvement in overall uterine condition.

In 2012, Christoffersen *et al*., evaluated the effect of immunomodulatory therapy [glucocorticoids and MCWF] on the endometrial gene expression of inflammatory cytokines in susceptible mares with induced infectious

endometritis. It was observed that treatment with MCWF did not modulate gene expression of inflammatory cytokines, however, it had a modulating effect on intrauterine fluid accumulations and it successfully cleared uterine pathogens in MCWF-treated mares.

The endometrial mRNA transcription patterns of five pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10 and TNFα) in mares that are resistant (RM) or susceptible (SM) to persistent post-breeding endometritis (PPBE) over three consecutive estrus periods were evaluated. The selection of RM and SM from a herd of 2,000 light Criollo cross-type mares aged between 6 and 16 years was based on results from uterine cytology, culture for bacteria and fungi, and a grade 1 or 2A uterine biopsy according to a previously established scoring system. Mares were also evaluated for evidence of intrauterine fluid, endometrial cytology and culture results 72 hours after intrauterine inoculation with
5 x$10^6$ cfu of *S. zooepidemicus*. Finally, the ability of the mares to become pregnant after two cycles of breeding with fertile stallions was assessed. From the above criteria, eight RM and eight SM mares were selected. The endometrial mRNA transcription patterns of IL-1β, IL-6, IL-8, IL-10 and TNFα were evaluated on SM and RM in three different experiments.

- Experiment 1: Determination of IL-1β, IL-6, IL-8, IL-10 and TNFα mRNA basal expression levels in SM and RM and the effect of estrus cycle on these cytokines.
- Experiment 2: Effects of artificial insemination (AI) with killed semen on IL-1β, IL-6, IL-8, IL-10 and TNFα mRNA expression in SM and RM (dead semen was used to study the inflammatory effect of semen deposition in the uterus while avoiding the otherwise superimposed effects of conception).
- Experiment 3: Effects of AI with killed semen plus Settle treatment on IL-1β, IL-6, IL-8, IL-10 and TNFα mRNA expression in SM and RM (this experiment was similar to experiment 2 except that the immunostimulant Settle was injected intravenously to mares at the time of AI).

Differences in IL-1β, IL-6 and TNFα mRNA expression between SM and RM in estrus 24 h following AI were not statistically significant, but expression levels of IL-8 were statistically higher in SM than in RM. In diestrus, mRNA expression of IL-1β (p=0.01), IL-8 (p=0.0001), IL-10 (p=0.02) and TNFα (p=0.02) was significantly higher in SM than in RM. When Settle was administered at the time of AI (semen-induced endometrial inflammation), there were no significant differences between mRNA transcription for IL-1β, IL-6 and TNFα between RM and SM either during estrus or during diestrus. Regarding the expression of IL-8 and IL-10, there was an observed statistically significant upregulation of IL-8 (p=0.28) and IL-10 (p=0.005) mRNA expression during estrus in SM compared to RM, but during diestrus there were no significant differences between mRNA transcription of both cytokines. When RM and SM mRNA expression levels of the five cytokines were compared between the AI and the AI + Settle groups, it was demonstrated that the administration of Settle resulted in a decrease in IL-1β, IL-6 and TNFα and a significant increase in IL-10 and IL-8 mRNA expression in SM during estrus compared to the same

From this study, it was demonstrated that Settle helps to restore the homeostatic local inflammatory mechanisms of the uterine environment and, consequently, assist in the prophylaxis of post-breeding endometritis in mares.

mares not receiving MCWF at time of AI. During diestrus, there was a non-significant decrease in expression of IL-8 and IL-10 in SM that received AI + Settle treatment as compared to the SM that received only AI (Fumuso *et al.,* 2003a; Fumuso *et al*., 2007). These results are in agreement with previous published data that has shown that mycobacterial cell wall components have an impact on cytokine regulation, for example, muramyl dipeptides have been shown to upregulate the production of IL-2, IL-4, IL-10, IL-12 and IFNγ (Gobec *et al*., 2004). As discussed previously, MCWFs have many distinct cell-wall components that may interact with different members of the toll-like receptor family, which are involved in the innate and adaptive immunological responses, hence the observed host response after MCWF administration.

## Future Research

In the equine, MCWF has been shown to be effective in treating both viral and bacterial infections and has also been shown to be effective in the treatment of equine sarcoid tumors. Due to the preclinical information that has been generated on MCWF formulations, it would be logical to conclude that an MCWF formulation may have some application in the control of migrating parasites (*Strongylus vulgaris*, *Parascaris equorum*).

In the canine, it has been shown that the MCWF technology can play a role in treating additional malignancies beyond mammary tumors. As discussed above, there have been studies showing the effectiveness of MCWF formulations to treat canine bladder transitional cell carcinoma, mast cell tumor (mastocytoma), salivary gland carcinoma, nasal squamous cell carcinoma, osteosarcoma, and lymphoma with encouraging results.

NovaVive has preliminary evidence of clinical efficacy using MCWF to treat malignancies in the cat.

In the bovine, in addition to the antibacterial effects of MCWF in the neonatal calf, it would seem feasible to evaluate MCWF in the prevention and treatment of other diseases, such as, bovine respiratory disease complex (BRDC) and mycoplasma infections, as well as the role of MCWF formulations to treat endometritis of the cow with the aim of improving conception rates.

## Concluding Remarks

The veterinary profession faces many difficult decisions. In the field of infectious disease, it is clear that, due to the current situation of antibiotic, anthelmintic and antiprotozoal resistance, the veterinarian must evaluate the role of alternative therapies to treat or prevent bacterial and viral insults. There is an obvious and increasingly important role for immunotherapeutic agents.

Veterinarians are important first-line practitioners in the developing 'one-health' paradigm. As such, and where possible, veterinarians should ensure that antibiotics are only used to treat bacterial infections and only those infections that are susceptible to the chosen antibiotic. Newer antibiotics should not be prescribed when an older antibiotic will be as effective. This is particularly true for β-lactam antibiotics such as cephalosporins. Antibiotics should not be prescribed when a non-antibiotic approach will be as effective!

The data collected from the use of MCWF over many years points to the fact that its non-specific immunotherapeutic abilities seem to restore the homeostasis of local inflammatory mechanisms via the regulation of cytokines to levels similar to those found in healthy animals. There is an opportunity for future research exploring the MCWF technology in the treatment of infectious diseases of different etiologies in other animal species.

As previously stated, MCWF exerts additive or synergistic effects with mechanisms of the innate immune system due to its influence on the production of interferons and cytokines. This synergistic effect augments the likelihood of MCWF killing invading pathogens in a timely manner, utilizing numerous antimicrobial mechanisms, such as, stimulation of cytokines, antimicrobial peptides, reactive oxygen species (oxidative burst) and reactive nitrogen intermediates (nitric oxide production).

Veterinarians are important first-line practitioners in the developing 'one-health' paradigm.

As such, and where possible, veterinarians should ensure that antibiotics are only used to treat bacterial infections and only those infections that are susceptible to the chosen antibiotic.

Newer antibiotics should not be prescribed when an older antibiotic will be as effective.

This is particularly true for β-lactam antibiotics such as cephalosporins.

Antibiotics should not be prescribed when a non-antibiotic approach will be as effective

## References


1. Alexandroff AB, Jackson AM, O'Donnell MA and James K. 1999. BCG immunotherapy of bladder cancer: 20 years on. Lancet, **353**, 1689-1694.
2. Alkemade Stanley J. 1989. Preliminary studies into the immunological effects of a purified mycobacterial cell fragment immunostimulant. Presented at the 11th Bain-Fallon Memorial Lectures, Australian Equine Veterinary Association.
3. Alkemade Stanley J, Buckley Thomas and McRae Graeme (Inventors). Immunostimulatory Bacterial Cell Wall Fractions. U.S. Patent No. 5,759,554 (June 06, 1998).
4. Barza MJ, Blum JH and Graeme-Cook FM. 1998. A 57-year-old man with fever and jaundice after intravesical instillation of Bacille Calmette-Guerin for bladder cancer. Case 29-1998. Case records of the Massachusetts general hospital. Weekly clinico-pathological exercises. N Engl J Medicine, **339**(12), 831-837.
5. Bernstein A, Piatti P, Gaggino OP, Schudel AA and Sadir AM. 1991. Enhancement of immune response elicited with FMD disease virus by an extract of *Mycobacterium* sp. Cell wall. Vaccine, **9**, 883-887.
6. Bogdan, C. 2001. Nitric oxide and the immune response. Nature Immunology, **2**, 907–916.
7. Chakravortty D and Hensel M. 2003. Inducible nitric oxide synthase and control of intracellular bacterial pathogens. Microbes Infect., **5**, 621–627.
8. Chedid L, Parant M, Parant F, Gustafson RH and Barger FM. 1972. Biological study of a non-toxic, water-soluble immunoadjuvant from mycobacterial cell wall. Proceedings of the National Academy of Science of the USA, **69**, 885-858.
9. Chin JL, Kadhim SA, Batislam E, Karlik SJ, Garcia BM, Curtis Nickel J and Morales A. 1996. Mycobacterium cell wall: an alternative to intravesical *Bacillus* Calmette Guérin (BCG) therapy in orthotopic murine bladder cancer. Journal of Urology, **156**, 1189-1193.
10. Christoffersen, M, Woodward EM, Bojesen AM, Petersen MR, Squires EL, Lehn-Jensen H and Troedsson MHT. 2012. Effect of immunomodulatory therapy on the endometrial inflammatory response to induced infectious endometritis in susceptible mares. Theriogenology, **78**, 991–1004.
11. Cleveland MG, Gorham JD, Murphy TL, Tuomanen E and Murphy KM. 1996. Lipoteichoic acid preparations of Gram-positive bacteria induce interleukin-12 through a CD14- dependent pathway. Infection and Immunology, **64**, 1906-1912.
12. Cormack S, Alkemade S and Rogan D. 1991. Clinical Study Evaluating a Purified Mycobacterial Cell Wall Extract for the Treatment of Equine Respiratory Disease. Equine Practice, 13(8).
13. Cortese V, Hankins K., Holland R and Syvrud K. 2013. Serologic responses of West Nile virus seronegative mature horses to West Nile virus vaccines. Journal of Equine Veterinary Science, **33**, 1101-1105.

14. Curtis GA, and Soloway MS. 1998. Complications of BCG in the treatment of superficial bladder cancer. Contemp Urolology, 48:53.
15. Dang LH, Bettegowda C, Huso DL, et al. 2001. Combination bacteriolytic therapy for the treatment of experimental tumors. Proc Natl Acad Sci USA, **98**, 15155-15160.
16. Engel MA and Neurath MF. 2010. Anticancer properties of the IL-12 family – Focus on colorectal cancer. Current Med. Chem., **17**(29), 3303-3308.
17. Fang FC. 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nature Rev. Microbiol., **2**, 820–832.
18. Filion MC and Phillips NC. 1997. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim Biophys Acta*, **1329,** 345–356
19. Filion MC, Lépicier P, Morales A and Phillips NC. 1999. *Mycobacterium phlei* cell wall complex directly induces apoptosis in human bladder cancer cells. Br J Cancer, **79**, 229-235.
20. Filion MC, Filion B, Reader S, Menard S and Phillips NC. 2000. Modulation of interleukin-12 synthesis by DNA lacking the CpG motif and present in a mycobacterial cell wall complex. Cancer Immunol Immunotherapy, **49**, 325-334.
21. Fumuso E., Giguère S, Wade J, Rogan D, Videla-Dorna I and Bowden R. 2003a. Endometrial IL-1beta, IL-6 and TNF-alpha, mRNA expression in mares resistant or susceptible to post-breeding endometritis: effects of estrous cycle, artificial insemination and immunomodulation. Vet Immunol Immunopathology, **96**, 31–41.
22. Fumuso E, Álvarez G, Bruno S, Videla-Dorna I, Wade J, Rogan D and Bowden R. 2003b. Non-specific immunomodulation at post-partum, improves uterine condition and fertility in mares. Proceedings, 8th World Equine Veterinary Association.
23. Fumuso E, Aguilar J, Giguère S, Rivulgo M, Wade J, and Rogan D. 2007. Immune parameters in mares resistant and susceptible to persistent post-breeding endometritis: Effects of immunomodulation. Vet. Immunol. And Immunopathology, **118**:30-39.
24. Gee K, Guzzo C, Che Mat NF, Ma W and Kumar A. 2009. The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. Inflamm. Allergy Drug Targets, **8**(1), 40-52.
25. Gobec S, Plantan I, Mravljak J, Wilson RA, Besra GS, Kekelj D. 2004. Phosphonate inhibitors of antigen 85C, a crucial enzyme involved in the biosynthesis of the *Mycobacterium tuberculosis* cell wall. Bioorg. Med. Chem. Lett, **4**:3559-3562.
26. Goubau S, Silversides DW, Gonzalez A, Laarveld B, Mapletoft RJ and Murphy BD. 1989. Immunization of cattle against modified peptides of gonadotropin releasing hormone conjugated to carriers: Effectiveness of Freund's and alternative adjuvants. Theriogenology, **32**(4), 557-567.
27. Griebel Phillip. 1999. Evaluation of the ability of mycobacterium cell wall fraction (MCWF) immunostimulant to alter blood leukocyte populations in newborn calves. Veterinary Infectious Disease

Organization (VIDO). Saskatoon, Saskatchewan, Canada. December 08, 1999.

28. Halpern BN, Biozzi G, Stiffel C and Mouton D. 1966. Inhibition of Tumour Growth by Administration of Killed *Corynebacterium parvum*. Nature, **212**, 853-854.
29. Indrigo J, Hunter RL and Actor JK. 2003. Cord factor trehalose 6,6- dimycolate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages. Microbiology, **149,** 2049-2059.
30. Johnson CS. 1991. Interleukin-1: therapeutic potential for solid tumors. Cancer Investigation, **11**, 600–608.
31. Kabbaj M and Phillips NC. 2001. Anticancer activity of Mycobacterial DNA: Effect of formulation as chitosan nanoparticles. Journal of Drug Targeting, **9**, 317-328.
32. Katila T. 1996. Uterine defense mechanisms in the mare. Anim. Reprod. Science, **42**, 197-204.
33. Kim IY, Smith C, Olivero J and Lapin SL. 2000. *Bacillus C*almette-*G*uérin induced peritonitis in a patient on dialysis. Journal of Urology, **163**, 237.
34. Ko H, Roszkowski W, Jeljaszewicz J and Pulverer G. 1981. Comparative study on the immunostimulatory potency of different *Propionibacterium* strains. Medical Microbiology and Immunology, 170(1), 1-9.
35. Korf J, Stoltz, A, Verschoor J, De Baetselier, P and Grooten, J. 2005. The *Mycobacterium tuberculosis* cell wall component mycolic acid elicits pathogen-associated host innate immune responses. Eur. J. Immunnology, **35**, 890–900.
36. Lamm D L, van der Meijden APM and Morales A. 1992. Incidence and treatment of complications of *bacillus* Calmette Guérin intravesical therapy in superficial bladder cancer. Journal of Urology, 147, 596.
37. Lancaster WD, Olson C and Meinke W. 1977. Bovine papillomavirus: presence of virus-specific DNA sequences in naturally occurring equine tumors. Proc. Natl. Acad. Sci. USA, **74,** 524–528.
38. LeBlanc M, Neuwirth L, Mauragis D, Klapstein E and Tran T. 1994. Oxytocin enhances clearance of radiocolloid from the uterine lumen of reproductively normal mares and mares susceptible to endometritis. Equine Veterinary Journal, **26**, 279 – 82.
39. Lowenstein CJ and Padalko E. 2004. iNOS (NOS2) at a glance. J. Cell Sciences, **117**, 2865–2867.
40. Lunardi M, Kussumoto de Alcântara B, Arellano Otonel RA, Borges Rodrigues W, Fernandes Alfieri A and Alcindo Alfieri A. 2013. Bovine papillomavirus type 13 DNA in equine sarcoids. J Clin Microbiology, **51**(7), 2167–2171.
41. MacMicking J, Xie QW and Nathan C. 1997. Nitric oxide and macrophage function. Annu. Rev. Immunology, **15**, 323–350.
42. Mallick BB, Kishore S, Daz SK and Garg A. 1985. Nonspecific immunostimulation against viruses. Comp. Immun. Microbiol. Infect. Disease, **8**(1), 53-63.
43. Mangieri J, Fiallos R, and Van der Linden I. 1995a. Tratamiento multidisciplinario (cirugia + quimioinmunoterapia) en el osteosarcoma (OSA) canino. Informe preliminar. (Multidisciplinary treatment

(surgery + chemoimmunotherapy) of canine osteosarcoma (OS). Preliminary report). Ciencia Veterinaria, **23**, 31-35.

44. Mangieri J. 1995. Estudio comparativo de distintos tratamientos no quirúrgicos para el sarcoma de Sticker (tumor venéreo transmissible) (Comparative study of the non-surgical treatments for TVT). Farmacologia y Terapeutica, 11:**57**, 105-116.
45. Mangieri J, Fiallos R, Van der Linden I. 1995b. Uso de la combinación quimio-immunoterapia para el tratamiento del tumor venéreo transmissible (TVT): preliminary report. Farmacologia y Terapeutica, 11:**57,** 134-141.
46. Mangieri J. 2003. Tratamiento del tumor venéreo transmisible genital del canino con quimioinmunoterapia (Chemotherapy treatment of canine transmissible venereal tumor (TVT). REDVET (Revista Electrónica de Veterinaria - http://www.veterinaria.org/revistas/redvet/n101003.html).
47. Mangieri J and Tondi A. 2003. Tratamiento del osteosarcoma apendicular canino (Treatment of canine appendicular osteosarcoma). REDVET (Revista Electrónica de Veterinaria – http://www.veterinaria.org/revistas/redvet/n111103.html).
48. Mangieri J, de Haro R, Rodrigues L, Kukolj V, Prunic B and Masic A. Safety profile of Mycobacterium Cell Wall Fraction (IMMUNOCIDIN®) following multiple intravenous administrations in healthy dogs. Proceedings, Veterinary Cancer Society Annual Conference, October 15-17, 2015. Tysons, Virginia, USA.
49. Mathé G, Kamel M, Dezfulian M, Halle-Pannenko O and Bourut C. 1973. An Experimental screening for "Systemic Adjuvants of Immunity" applicable in cancer immunotherapy. Cancer Research, **33**, 1987-1997.
50. Means TK, Jones BW, Schromm AB, Shurtleff BA, Smith JA, Keane J, Golenbock DT, Vogel SN, and Fenton MJ. 2001. Differential effects of a Toll-like receptor antagonist on Mycobacterium tuberculosis-induced macrophage responses. J Immunology, **166**, 4074-4082.
51. Morales A, Nickel JC, Downey J, Clark J and Van der Linden I. 1995. Immunotherapy of an experimental adenocarcinoma of the prostate. Journal Urology, **153**, 1706-1710.
52. Morales A, Chin JL, and Ramsey EW. 2001. Mycobacterial cell wall extract for treatment of carcinoma *in situ* of the bladder. J Urology, **166**, 1633-1637.
53. Murphy JM, Severin GA, Lavach JD, Hepler DI and Lueker DC. 1979. Immunotherapy in ocular equine sarcoids. JAVMA, **174**, 269.
54. Nair N, Kasai T and Seno M. 2014. Bacteria: Prospective savior in battle against cancer. Anticancer Research, **34**(11), 6289-6296.
55. Nash David. 2003. Effects of Immunoboost® on Health and Performance of Holstein Steer Calves. Technical Report. Bioniche Animal Health USA, Inc., Bogart, GA 30622.
56. Neville ME and Pezzella KM. 1994. Anti-tumour effects of interleukin 1 beta: in vivo induction of immunity to B16 melanoma, a non-immunogenic tumour. Cytokine, **6**, 310–317.
57. Nosky and Worthington, 1996. Effects of prophylactic administration of a nonspecific immunotherapeutic

on the performance of Hutch-reared calves.

58. Ostlund EN, Crom RL, Pedersen DD, Johnson DJ, Williams WO, Schmitt BJ. 2001. Equine West Nile encephalitis, United States. Emerg. Infect Disease, **7**, 665-669.
59. Otten N, von Tscharner C, Lazary S, Antczak DF and Gerber H. 1993. DNA of bovine papillomavirus type 1 and 2 in equine sarcoids: PCR detection and direct sequencing. Arch. Virology, **132**, 121–131.
60. Patyar S, Joshi R, Prasad Byrav DS *et al*. 2010. Bacteria in cancer therapy: A novel experimental strategy. J Biomed Sciences, **17**, 21.
61. Pawelek J, Low K and Bermudes D. 1997. Tumor-targeted Salmonella as a novel anticancer vector. Cancer Research, **57**, 4537-4544.
62. Radtke AL and O'Riordan MX. 2006. Intracellular innate resistance to bacterial pathogens. Cell. Microbiology, **8**, 1720–1729.
63. Reader S, Ménard S, Filion B, Filion MC and Phillips NC. 2001. Pro-apoptotic and immunomodulatory activity of a Mycobacterial cell wall-DNA complex towards LNCaP prostate cancer cells. The Prostate, **49**, 155-165.
64. Rodrigues L, Masic A, Alkemade S, Kukolj V, Martins M and Montini E. Mycobacterium Cell Wall Fraction (IMMUNOCIDIN®) for canine transitional cell carcinoma treatment. Proceedings, Veterinary Cancer Society Annual Conference, October 15-17, 2015. Tysons, Virginia, USA.
65. Rogan D, E. Fumuso E, Rodríguez J, Wade J and Sanchez Bruni SF. 2007. Use of a Mycobacterial Cell Wall Extract (MCWE) in Susceptible Mares to Clear Experimentally Induced Endometritis with Streptococcus zooepidemicus. Journal of Equine Veterinary Sciences, 112-116.
66. Strohmeier GR and Fenton MJ. 1999. Roles of lipoarabinomannan in the pathogenesis of tuberculosis. Microbes Infect, **1**, 70.
67. Sundberg JP, Burnstein T and Page EH. 1977. Neoplasms of Equidae. J. Am. Vet. Med. Association, **170**, 150–152.
68. Tome Y, Zhang Y, Momiyama M, Maehara H, Kanaya F, Tomita K, Tsuchiya H, Bouvet M, Hoffman RM and Zhao M. 2013. Primer dosing of *Salmonella* Typhimurium AR-1 potentiates tumor-targeting and efficacy in immunocompetent mice. Anticancer Research, **33**(1), 97-102.
69. Troedsson MHT. 1995. Comparative treatment of mares susceptible to chronic uterine infection. American Journal of Veterinary Research, **56**, 468–472.
70. Troedsson MHT. 1997. Therapeutic considerations for mating-induced endometritis. Pferdeheilkunde, **13**, 516–520.
71. Troedsson MHT. 1999. Uterine clearance and resistance to persistent endometritis in the mare. Theriogenology, **52**, 461-471.
72. Velasco-Velazquez MA, Barrera D, Gonzalez-Arenas A, Rosales C and Agramonte-Hevia J. 2003.

Macrophage-Mycobacterium tuberculosis interactions: role of complement receptor 3. Microb Pathogens, **35**, 125-131.

73. Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ and Folkman J. 1995. Inhibition of angiogenesis *in vivo* by interleukin 12. Journal Natl Cancer Institute, **87**(8), 581–586.

74. Wade Jose and Stanley J. Alkemade. 1997. Method for stimulation of reproductive performance. U.S. Patent No. 5,632,995 (May 27, 1997).
75. Watson E. 2000. Post-breeding endometritis in the mare. *In: Proceedings of the 14th International Congress on Animal Reproduction*, 2-6 July 2000, Stockholm. Elsevier, pp221-232.
76. Yarkoni E, Hunter JT, Sukumar S and Rapp HJ. 1982. Active specific immunotherapy of Guinea pigs with visceral tumor implants, Cancer Immunol Immunotheraphy, **12**, 273-275.
77. Yarkoni E and Rapp HJ. 1980. Immunotherapy of experimental cancer by intralesional injection of emulsified nonliving mycobacteria: comparison of *Mycobacterium bovis* (BCG), *Mycobacterium phlei*, and *Mycobacterium smegmatis*. Infect Immunology, **28**, 887.
78. Young SL, Murphy M, Xing WZ, Harnden P, O'Donell MA, Keith J., Poulam MP, Selby PJ and Jackson AM. 2004. Cytokine-Modified *Mycobacterium smegmatis* as a Novel Anticancer Immunotherapy. Int. J. Cancer, **112**, 653–660.
79. Zbar B, Canti G, Ashley MP, Rapp HJ, Hunter JT and Ribi E. 1979. Eradication by immunization with mycobacterial vaccines and tumor cells of microscopic metastases remaining after surgery. Cancer Research, **39**, 1597.
80. Data on file, Study ID # BIS-40
81. Data on file, Study ID # Regressin, MCW Research Summary.
82. Data on file, Study ID # BEC-13
83. Data on file, Study ID # E.I.S.-2
84. Data on file, Study ID # E.I.S.-3
85. Data on file, Study ID # Equimune potentiation of a commercial West Nile virus vaccine in horses
86. Data on file, Study ID # Efficacy of Mycobacterial Cell Wall Extract (MCWE) in the treatment of osteosarcoma in dogs
87. Data on file, Study ID # ER-1 Equine sarcoids.
88. Data on file, Study ID # VR-C-LIC-I-1989.
89. Data on file, Study ID # NV-C-SGS-I-2015.
90. Data on file, Study ID # NV-C-L-I-2015.