Hyperimmune Canine Plasma (Caniplas®) to *Escherichia coli (E. coli* J5) for use in Septicaemia and Inflammation

R. B. Brandon and R. P. Wilson

R. B. Brandon
BVSc (Hons) PhD MBA
rbrandon@plasvacc.com

R. P. Wilson
BVSc (Hons)
rosswilson@plasvacc.com

Plasvacc Australia
(Plasvacc)
6066 Cunningham Hwy
Kalbar
Queensland
Australia 4309
61-7-54637600

Objectives – To develop an effective *Escherichia coli* (*E. coli*) vaccine that engenders a strong and measurable humoral response in canine plasma donors. To develop an assay to determine potency of plasma derived from vaccinated canine donors. To determine efficacy of hyperimmune plasma to *E. coli* in models of septicaemia and inflammation. To identify new active components of hyperimmune plasma derived from vaccinated canine donors.

Procedure – A proprietary vaccine formulation of *E. coli* (J5) was developed and tested on a colony of 50 greyhound donors. Antibody end-point titres to whole *E. coli* were determined using a developed J5 EIA on plasma from Plasvacc dogs, healthy dogs, and canine plasma presenting at a pathology laboratory. Effects of Caniplas® were measured in a canine model of septicaemia and in a rat model of inflammation. Quantities of soluble tumour necrosis factor receptor (sTNFR) in canine serum were determined using an anti-canine sTNFR antibody in an immunofluorescent assay.

Results – A proprietary, safe and effective *E. coli* (J5) vaccine has been developed for use on canine plasma donors as part of the production of Caniplas®. Plasvacc donor dogs have a higher average plasma anti-J5 titre compared to healthy dogs but lower than that of plasma presenting to a pathology laboratory. Caniplas® has measurable anti-TNF activity when used in a model of septicaemia in dogs and in a rat skin pouch model of inflammation. The anti-TNF activity can in part be explained by the fact that Plasvacc donor dog serum has higher levels of sTNFR than serum derived from unvaccinated dogs. Immunoglobulins in plasma are very stable and tolerant of long periods of frozen and refrigerated storage.

Conclusions and Clinical Relevance – Plasvacc has a highly effective and proprietary *E. coli* vaccine for use in generating canine plasma with high levels of anti-J5 antibody and levels of sTNFR higher than unvaccinated dogs. Results using clinical models of endotoxaemia and inflammation suggest that Caniplas® can be used to effectively treat conditions where endotoxaemia, increased inflammation and TNFα play a role in pathogenesis. Caniplas® has demonstrable long-term immunoglobulin stability at both frozen and refrigerated temperatures.

Introduction

It is known that septicaemia and / or bacterial translocation from the gut play a key role in the pathogenesis of a number of important canine veterinary conditions including;

- Parvovirus infection[1],
- Gastric atony[2],
- Gastric dilatation/volvulus[3],
- Acute pancreatitis[4],
- Wounds, burns and haemorrhagic shock[5,6],
- Ischaemia and hypoxia[7,8,9], and
- Pyometra[10].

Endotoxin is thought to be responsible for most, if not all, of the features of Gram-negative septicaemia, which is largely mediated by cytokines and acute phase proteins[11].

In an effort to allay the effects of endotoxin researchers have studied the effects of immunization with an Rc mutant strain of *E. coli* O111:B4, or J5. This strain of *E. coli* contains lipopolysaccharide (LPS) where the core region is not protected by the O antigen. LPS is also referred to as endotoxin. J5 immunization has been demonstrated to have the following effects *in vivo*:

- Prevention of sepsis in rats[12],
- Increased clearance of gram-negative bacteria in mice, and
- Improved outcome in experimental polymicrobial intra-abdominal sepsis in mice[13],
- Reduced clinical signs, culling and death from clinical mastitis in cows[14],
- Provide cross-protection to a number of other bacteria including a number of *Staphylococcal*, *Serratia*, *Psuedomonas* and *Klebsiella* species[15].

Further, high levels of circulating plasma antibodies (either IgG or IgM) to endotoxin have been demonstrated to:

- Correlate to survival of human sepsis patients[16,17],
- Reduce LPS-induced cytokine generation in whole human blood[18],
- Correlate to the presence of experimentally-induced colitis in rats[19],
- Correlate to severity of acute pancreatitis in humans[20],
- Be higher in convalescent children who have had intussusception[21],
- Correlate to long-term survival of humans who have undergone coronary artery bypass graft surgery[22],
- Inversely correlated to adverse post-operative outcome in cardiac surgery[23],
- Attenuate portal endotoxaemia in an experimental model of lower limb ischaemia-reperfusion in pigs[24].

Therefore, prior exposure to endotoxin through the development of antibodies appears to protect animals from septicaemia, conditions that lead to septicaemia and adverse clinical signs associated with septicaemia.

Such protection can not only be gained from direct immunization but through passive transfer of immunoglobulin through the use of intravenous immunoglobulin (IVIG in humans) or hyperimmune plasma in animals. For example, IVIG prepared from pooled human plasma is used in human medicine in the treatment of a wide range of conditions, from replacement therapy to sepsis to neuroimmunological diseases[25] – for a full range of clinical uses of IVIG see Table I on page 234 in this reference. The mode of action of IVIG is complex, through multiple modes of action, and is thought to act synergistically on Fc receptors, complement, dendritic cells and T and B cells[26]. Plasma therapy is also useful in veterinary medicine[27] and has been used successfully in sepsis in horses as measured by reduced time to recovery and overall survival and is indicated for use in sepsis in dogs[28,29,30,31].

Plasvacc has been researching the effectiveness of J5 hyperimmune plasma in septicaemia and bacterial translocation because they are a complicating factor in many conditions in veterinary medicine and are often the ultimate cause of death.

This white paper describes:

- The development and use of a proprietary J5 vaccine,
- The development of an EIA (Enzyme ImmunoAssay) to measure donor response to J5,
- Experimental use of hyperimmune J5 plasma in endotoxaemia and inflammation models,
- Identification of active molecules in hyperimmune J5 plasma,
- Stability studies.

Such work aims to provide the small animal veterinarian with professional confidence in the knowledge they are using an effective, potent, appropriately labelled and safe product in conditions where septicaemia is a potential complicating factor.

Materials and Methods

Vaccine
Plasvacc has developed a proprietary vaccine formulation of an Rc (rough coat) mutant strain of *E. coli* O111:B4, or J5. This strain is known to lack expression of the O antigen and was originally isolated from a clinical case by The University of Queensland Veterinary School Microbiology Department. Donor dogs were immunised with multiple doses of vaccine and titres of anti-J5 antibody were monitored by Enzyme Immunoassay (EIA – see below).

Potency Assay (EIA)
An EIA was developed to measure specific antibodies to J5 in canine plasma. Briefly, reagents used in the EIA included known quantities of J5 as the antigen bound to the plate and goat anti-dog IgG (H+L) conjugated to Horse Radish Peroxidase (HRP) as the reporter molecule. Results were expressed as an end-point titration at twice background level (e.g. no recordable optical density at a plasma dilution of 1:10,000 is reported as 10,000 – the higher the dilution the larger the quantity of anti-J5 antibodies).

Potency Testing
The anti-J5 titres of plasma from J5 vaccinated Plasvacc dogs were compared to a cohort of healthy local dogs (obtained using owner consent) and to a cohort of animals whose blood presented at a local pathology laboratory for testing. The latter cohort of dogs' clinical condition and owners' identity were not made known to Plasvacc.

Endotoxaemia Model
A canine model of septicaemia using intravenous administration of LPS was used under the direction of The University of Queensland animal ethics committee. Briefly, anaesthetised dogs were administered either Caniplas®, fresh frozen plasma or saline one hour prior to challenge with LPS. Provision was made for the measurement of central venous pressure, heart rate and respiratory rate. Blood samples were taken -60, 0, +5, +15, +30, +60, +120, +180 minutes relative to LPS injection for haematology, biochemistry and anti-TNFα analysis.

Inflammation Model
A rat subcutaneous skin pouch model of inflammation was used also under the direction of The University of Queensland and University of Southern Queensland animal ethics committees. Briefly, a skin pouch was created in anaesthetised rats by subcutaneous injection of air and then inflammation was created by the injection of an irritant (monosodium urate, MSU). Rats were pre-treated with either Caniplas®, fresh frozen plasma, Etanercept[32], saline, or carprofen prior to injection of MSU into the skin pouch. Samples of fluid were taken from the pouches at 1, 6, 12, 24 and 48 hours post-irritant for haematology and anti-TNFα analysis.

Measurement of Anti- TNFα Activity
Anti-TNFα bioactivity was measured on serum samples from the septicaemia study and on sera from Plasvacc dogs (HFS) and healthy dogs (FFS) using a TNFα-sensitive fibroblast mouse cell line (L929). Briefly, cell survival of monolayer L929 cells was measured following exposure to various dilutions of canine TNFα alone or when mixed with representative sera. Serum was used because fibrinogen in plasma interferes with results obtained using this assay.

Measurement of TNF
TNFα concentration was determined in the rat pouch fluid samples using a commercial ELISA kit (R&D Systems Inc.)

Measurement of sTNFαRI
Soluble tumour necrosis factor receptor 1 (sTNFRI) was measured in Caniplas® (HFP) and Fresh Frozen Plasma (FFP) using an immunofluorescent assay. Briefly, plasma allowed to dry on a microscope slide was reacted firstly with rabbit anti-canine sTNFαRI antibody followed by FITC-labelled goat anti-rabbit IgG. Images were recorded using a fluorescent microscope fitted with a camera.

Stability
Various batches of plasma in PVC bags and in the same volume as sold product were tested for total immunoglobulin G (IgG) levels following being subjected to:

- Freezing (-22^{0}C) for up to 4 years,
- Refrigeration (4^{0}C) for up to 108 days.

Total IgG was determined by Radial Immunodiffusion (RID).

APVMA and GMP
Plasvacc Caniplas® is produced under GMP, and is licensed through the Australian Pesticides and Veterinary Medicines Authority (APVMA) for sale through veterinarians only.

All dogs are tested for blood type and various diseases prior to entry to quarantined premises and on an on-going basis. Plasma is guaranteed to be: derived from healthy dogs, sterile, free of disease, have minimal cell counts, and to meet potency standards.

GMP licensing through the APVMA means Plasvacc is regularly audited by independent auditors acting on behalf of the APVMA. A license can be suspended at any time if production standards are not met.

Results

Potency Testing

The plasma of three groups of dogs was tested for the end-point titre of anti-J5 antibodies using the developed EIA. The groups consisted of blood from 1). Clinically healthy dogs, 2). Plasvacc dogs, 3). Dogs whose blood presented at a local pathology laboratory. Average anti-J5 end-point titres for these groups are presented in the following table.

Table 1

Average anti-J5 end-point titres for three groups of dogs

Healthy	Plasvacc	Pathology Lab.
8,800	13,400	16,300

For healthy dogs the anti-J5 titre ranged from 1,200 (a pup) to >51,200 (an old dog). For Plasvacc and Pathology Lab. dogs it ranged from 3,200 to >51,200. The average anti-J5 titre for Plasvacc dogs falls between clinically healthy dogs and Pathology Lab. dogs.

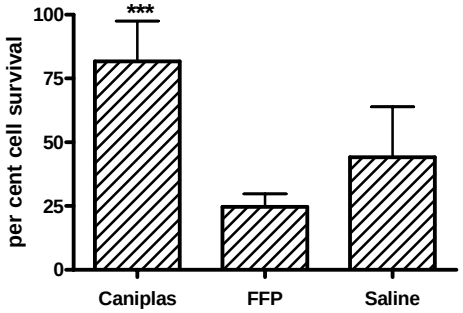
Endotoxaemia Model

Infusion of Caniplas® compared to fresh frozen plasma or saline prior to LPS challenge did not have any significant effect on the central venous pressure, heart rate, respiration rate, activated clotting time, haematocrit or numbers of circulating leukocytes.

However, the anti- TNFα activity, as measured by L929 assay and reported as % cell survival, in sera taken 120 minutes post-LPS challenge in dogs transfused with Caniplas® was significantly higher than in sera taken from those dogs transfused with either fresh frozen plasma or saline (see Figure 1 below).

Figure 1

Measure of cytotoxic activity (represented as % cell survival of L929 cells) present in sera of dogs subjected to a bolus infusion of either Caniplas® (n = 6), FFP (Fresh Frozen Plasma from healthy unvaccinated dogs) (n = 4) or saline (n = 4). Serum was taken 120 min post i.v. LPS. Serum from dogs infused with Caniplas® had a significantly higher % cell survival (reduction in cytotoxic activity of TNFα) compared to serum taken from those dogs receiving FFP and saline infusions (p<0.0001).

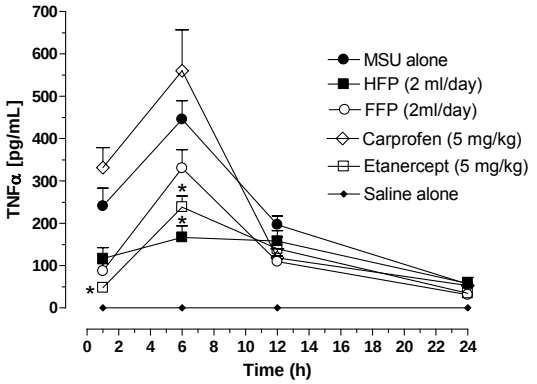


Inflammation Model

Fluids taken from skin pouches of rats pre-treated subcutaneously with a variety of substances, including Caniplas®, were measured for TNFα concentration. The results are displayed in Figure 2. Pre-treatment of rats with Plasvacc's Caniplas® has comparable effects to that of Etanercept with respect to reducing the concentration of TNFα present in rat skin pouches six hours following the injection of MSU.

Figure 2

This figure illustrates the effect of canine hyperimmune frozen plasma (Caniplas®) (HFP) and fresh frozen plasma (FFP) prophylactic subcutaneous infusions on TNFα levels induced by MSU injection in a rat pouch model of inflammation. The figure shows that HFP, but not FFP significantly depresses the level of TNFα present in the pouch exudates (p<0.05). The figure also shows that HFP and Etanercept have comparable effects on depression of TNFα levels, whilst the NSAID carprofen does not reduce levels of the inflammatory cytokine.



Measurement of Anti- TNFα and sTNFαRI

Anti-TNFα activity and sTNFαRI were measured in sera from Plasvacc dogs (Hyperimmune Frozen Serum - HFS) and sera from healthy unvaccinated dogs (Fresh Frozen Serum - FFS). Figure 4 shows results of the L929 anti-TNFα biological activity assay. Because of the anti-TNFα activity in HFS derived from Plasvacc dogs a significantly greater percent of L929 cells survive in the presence of canine TNFα compared to FFS.

Figure 4

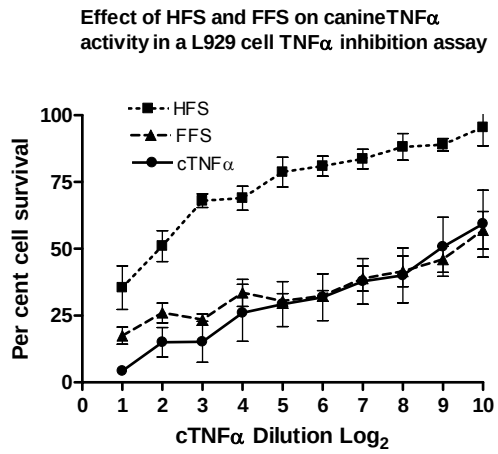
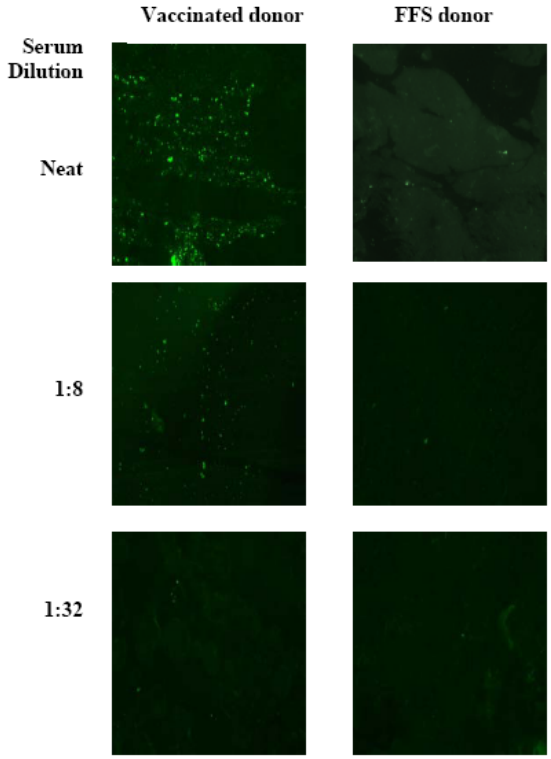


Figure 5 shows the results of an immunofluorscent assay of Plasvacc donor serum and serum derived from a healthy dog (FFS). Dose response-related reactivity with an anti-canine sTNFRI antibody suggests that Plasvacc donor serum contains sTNFRI in higher levels than that of healthy dog serum.

Figure 5

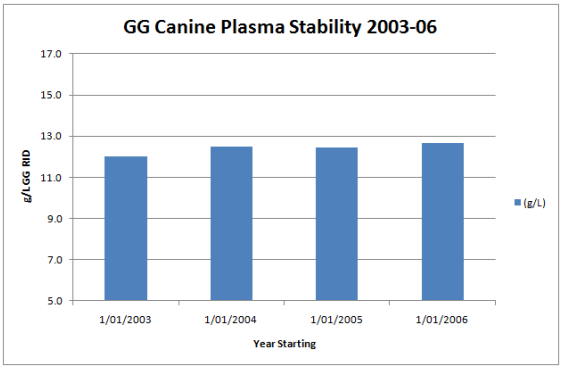
Fluorescent microscope images of slides with various dilutions of Plasvacc donor serum (neat, 1:8, 1:32) compared to Fresh Frozen Serum from a healthy dog reacted with a polyvalent anti-canine sTNFR1 antibody. Reactivity with neat Plasvacc donor serum suggests the presence of sTNFR1. The figure also shows that there are higher levels of reactivity to sTNFR1 present in Plasvacc vaccinated dog serum compared to FFP donor serum and that there is a dilution dose relationship.



Stability

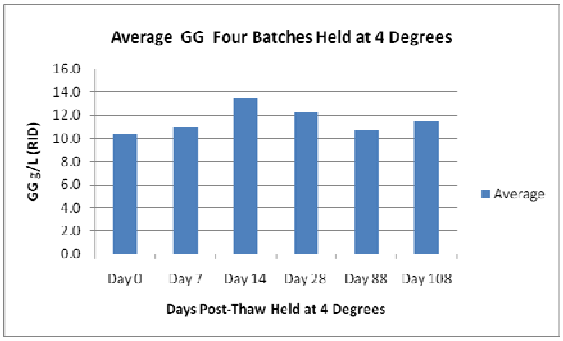
Forty-four batches of Caniplas® held frozen for up to four years were tested for total immunoglobulin G (IgG) using Radial Immunodiffusion (RID). Figure 6 shows the average total immunoglobulin (GG) levels obtained for those batches tested for the years 2003 through to 2009. It can be seen that immunoglobulin concentration remains stable when plasma is frozen in its original container for at least four years.

Figure 6
Summary of the average immunoglobulin (GG) level measured by radial immunodiffusion in grams/litre for 44 batches of Caniplas® stored frozen for up to four years between 2003 and 2006.



Four batches of Caniplas® held at 4^0C (refrigerator) for up to 108 days were tested for total immunoglobulin G (IgG) using Radial Immunodiffusion (RID). Figure 7 shows the average total immunoglobulin (GG) levels obtained. It can be seen that immunoglobulin concentration remains stable when plasma is kept refrigerated for up to 108 days.

Figure 7
Average immunoglobulin levels for four batches of Caniplas® thawed and then kept refrigerated for various periods.



Discussion

Anti-J5 antibody potency testing of Plasvacc dogs, healthy dogs and dog blood presenting at a pathology laboratory showed that the average anti-J5 antibody levels in Plasvacc dogs falls between that of healthy dogs and sick dogs. The clinical condition of the pathology laboratory dogs was not revealed to Plasvacc so it is only assumed that most of these dogs were sick. This result suggests that the Plasvacc J5 vaccine is a potent stimulator of anti-J5 antibodies in donor dogs. The vaccine has no side effects and all dogs remain healthy following vaccination.

In a canine endotoxaemia model, where dogs are infused intravenously with LPS, prior infusion of Caniplas® results in measurable anti-TNFα activity in serum samples taken 120 minutes following LPS challenge. There was no effect on central venous pressure, heart rate, respiration rate, activated clotting time, haematocrit or numbers of circulating lymphocytes. It is well known that intravenous injection of LPS is not a perfect model to study clinical evaluation of therapies in endotoxaemia or septicaemia[33]. However, these results suggest that Caniplas® contains factor(s) that allay the action of TNFα in dogs exposed to endotoxin. Further investigations of the anti-TNFα activity of Caniplas® were conducted in a model of inflammation in rats.

Quantities of TNFα in fluid taken from rat skin pouches six hours following the introduction of MSU were significantly reduced in rats pre-treated with Caniplas® and Etanercept when compared to rats pre-treated with carprofen and fresh frozen plasma. Again, this suggests that Caniplas® contains factor(s) that allay the action of, or reduce the expression of, TNFα in rats with inflammation.

Direct anti- TNFα activity was demonstrated in serum taken from Plasvacc donor dogs using the L929 assay. Significantly higher per cent L929 cell survival (anti-TNFα activity) exists in serum of Plasvacc donor dogs compared to serum derived from healthy dogs when using canine TNFα.

Investigation into the factor(s) in Plasvacc donor dog serum that contribute to TNFα antagonism included the use of an immuofluorescent assay and an anti-canine sTNFR antibody. This assay conclusively demonstrated the following:

- Plasvacc donor dog serum contains sTNFR,
- Plasvacc donor dog serum contains much higher levels of sTNFR when compared to serum from healthy dogs not exposed to the J5 vaccine,
- The level of sTNFR is titratable.

Taken it is entirety these data suggest that immunisation of donor dogs with Plasvacc's proprietary J5 vaccine induces high levels of plasma anti-J5 antibodies and anti-TNFα activity and that the anti-TNFα activity is in part due to increased levels of soluble tumour necrosis factor receptor.

Immunoglobulins are known to be very stable molecules in purified form or in plasma. Plasvacc has conducted studies over 7 years demonstrating that canine plasma is stable for long periods when stored at -20^{0}C or below and for up to 108 days at 4^{0}C. Despite these findings, Plasvacc strongly recommends following the storage guidelines provided on the label and disposing of plasma that has passed expiry date.

Conclusion

Plasvacc has a highly effective and proprietary *E. coli* vaccine for use in generating canine plasma with high levels of anti-J5 antibody and levels of sTNFR higher than unvaccinated dogs.

In conjunction with prior scientific literature these results suggest that Caniplas® can be used to effectively treat conditions where endotoxaemia, increased inflammation and TNFα play a role in pathogenesis.

These advances, along with Plasvacc's continued quality assurance and product registration, ensure that small animal veterinarians are provided with high quality product of known activity.

Acknowledgements
Plasvacc acknowledges the expert scientific, technical and professional work conducted by staff at both The University of Southern Queensland and The University of Queensland including: Professor Michael Kotiw, Dr Michael Morgan, Professor Ian A. Shiels, and Professor Stephen M. Taylor.

[1] Otto CM, Drobatz K, Soter C. Endotoxemia and tumor necrosis factor in clinical canine parvoviral enteritis. J Vet Intern Med; 11:65–70. 1997.
[2] Woosley KP. The problem of gastric atony. Clin Tech Small Anim Pract. Feb;19(1):43-8. 2004.
[3] Winkler KP, et al. Bacteremia and bacterial translocation in the naturally occurring canine gastric dilatation-volvulus patient. J Am Anim Hosp Assoc. Jul-Aug;39(4):361-8. 2003.
[4] Qin HL, et al. Early intrajejunal nutrition: bacterial translocation and gut barrier function of severe acute pancreatitis in dogs. Hepatobiliary Pancreat Dis Int. Feb;1(1):150-4. 2002.
[5] Yao YM, et al. Gut-derived endotoxemia and multiple system organ failure following gunshot wounds combined with hemorrhagic shock: an experimental study in the dog. J Trauma. 1995 May;38(5):742-6.
[6] Huang, Y.S. et al. Serial experimental and clinical studies on the pathogenesis of multiple organ dysfunction syndrome (MODS) in severe burns. Burns. 24, 706-716. 1998.
[7] Bibbo C, et al. Bacterial translocation after mesenteric ligation in dogs. J Invest Surg. 1996 Jul-Aug;9(4):293-303.
[8] Lelli JL Jr, et al. Hypoxia-induced bacterial translocation in the puppy. J Pediatr Surg. Aug;27(8):974-81. 1992.
[9] Zhi-Yong S, et al. Bacterial translocation and multiple system organ failure in bowel ischemia and reperfusion. J Trauma. Feb;32(2):148-53. 1992.
[10] Fantoni DT, et al. Intravenous administration of hypertonic sodium chloride solution with dextran or isotonic sodium chloride solution for treatment of septic shock secondary to pyometra in dogs. J Am Vet Med Assoc. Nov 1;215(9):1283-7. 1999.
[11] Neilly et al. Endotoxaemia and cytokine production in experimental colitis. Brit J Surg. 82: 1479-1482. 1995. See abstract, page 1479 and Table 2 on page 1480.
[12] Cross et al. Development of an anti-core lipopolysaccharide vaccine for the prevention and treatment of sepsis. Vaccine. 22(7): 812-817. 2004. See page 814.
[13] Opal et al. Active immunization with a detoxified endotoxin vaccine protects against lethal polymicrobial sepsis: its use with CpG adjuvant and potential mechanisms. J Inf Dis. 192(12): 2074-2080. 2005. See conclusions on page 2074.
[14] Wilson et al. Comparison of J5 vaccinates and controls for incidence, etiological agent, clinical severity, and survival in the herd following naturally occurring cases of clinical mastitis. J Dairy Sci. 90(9): 4282-4288. See last sentence of abstract on page 4282.
[15] Chaiyotwittayakun et al. Hyperimmunization of steers with J5 Escherichia coli bacterin: effects on isotype-specific serum antibody responses and cross reactivity with heterogenous gram-negative bacteria. J Diary Sci. 87: 3375-3385. 2004. See page 3375 in Abstract.
[16] Maury et al. Circulating endotoxin and antiendotoxin antibodies during severe sepsis and septic shock. J Crit Care. 18(2): 115-120. 2003. See page 115.
[17] Goldie et al. Natural cytokine antagonists and endogenous antiendotoxin core antibodies in sepsis syndrome. JAMA. 274(2): 172-177. 1995. See page 5.

[18] Cross et al. Development of an anti-core lipopolysaccharide vaccine for the prevention and treatment of sepsis. Vaccine. 22: 812-817. 2004. See abstract on page 812.

[19] Neilly et al. Endotoxaemia and cytokine production in experimental colitis. Brit J Surg. 82: 1479-1482. 1995. See abstract, page 1479 and Table 2 on page 1480.

[20] Penalva et al. A study of intestinal permeability in relation to the inflammatory response and plasma endocab IgM levels in patients with acute pancreatitis. J Clin Gastroenterol. 38(6): 512-517. 2004. See Results on page 512.

[21] Willetts et al. Endotoxin, cytokines and lipid peroxides in children with intussusceptions. Brit J Srug. 88: 878-883. 2001. See Results on page 878.

[22] Moretti et al. Effects of decreased preoperative endotoxin core antibody levels on long-term mortality after coronary artery bypass graft surgery. Arch Surg. 141: 637-641. 2006. See Results on page 637.

[23] Bennett-Guerrero et al. Relationship of preoperative antiendotoxin core antibodies and adverse outcomes following cardiac surgery. JAMA. 277(8): 646-650. See page 2 in Conclusion.

[24] Harkin et al. Anti-endotoxin hyperimmune globulin attenuates portal cytokinaemia, phagocytic cell priming, and acute lung injury after lower limb ischaemia-reperfusion injury. Eur J vasc Surg. 33: 330-339. 2007. See Conclusions on page 330.

[25] Negi et al. Intravenous immunoglobulin: an update on the clinical use and mechanisms of action. J Clin Imm. 27(3): 233-245. 2007.

[26] See reference 19. Negi et al. Intravenous immunoglobulin: an update on the clinical use and mechanisms of action. J Clin Imm. 27(3): 233-245. 2007. See page 237 and Figure 1 on page 238.

[27] Rozanski. Transfusion medicine in veterinary emergency and critical care medicine. Clin Tech Sm An Pract. 19(2): 83-87. 2004.

[28] Peek et al. Prognostic value of clinicopathological variables obtained at admission and effect of antiendotoxin plasma on survival in septic and critically ill foals. J Vet Intern Med. 20: 569-574. 2006. See abstract page 569.

[29] Spier et al. Protection against clinical endotoxemia in horses by using plasma containing antibody to an Rc mutant E coli (J5). Circ Shock. 28(3): 235-248. 1989. Abstract only.

[30] Callan et al. Canine red blood cell transfusion practice. J Am An Hosp Assoc. 32: 303-311. 1996. Abstract only. As referred to in Rozanski et al on page 86.

[31] Crowley, J.P et al. Effects of plasma administration on gram negative shock in granulocytopenic dogs. Circulation and Shock. 26, 287-295. 1988.

[32] Etanercept is a recombinant fusion protein of soluble tumour necrosis factor receptor and the Fc component of immunoglobulin G used in autoimmune disease to reduce the effects of TNFα. Also called Enbrel and produced by Amgen and Wyeth.

[33] Otto CM. Clinical trials in spontaneous disease in dogs: a new paradigm for investigations of sepsis. J Vet Emerg Crit Care. 17(4): 359 – 367. 2007.