Biomarkers in canine parvovirus enteritis

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Abstract

Canine parvovirus(CPV) enteritis has, since its emergence in 1978, remained a common and important cause of morbidity and mortality in young dogs. The continued incidence of parvoviral enteritis is partly due to the virus' capability to evolve into more virulent and resistant variants with significant local gastrointestinal and systemic inflammatory sequelae. This paper reviews current knowledge on historical-, signalment-, and clinical factors as well as several haematological-, biochemical- and endocrine parameters that can be used as diagnostic- and prognostic biomarkers in CPV enteritis. These factors include season of presentation, purebred nature, body weight, vomiting, leukopaenia, lymphopaenia, thrombocytopaenia, hypercoagulability, hypercortisolaemia, hypothyroxinaemia, hypoalbuminaemia, elevated C-reactive protein and tumour necrosis factor, hypocholesterolaemia and hypocitrullinaemia). Factors contributing to the manifestations of CPV infection are multiple with elements of host, pathogen, secondary infections, underlying stressors and environment affecting severity and outcome. The availability of several prognosticators has made identification of patients at high risk of death and their subsequent targeted management more rewarding.

Introduction

A **biomarker** can broadly be defined as an indicator of a biological state that is objectively measured and evaluated as a marker of physiological or pathological processes. In the context of CPV infection these biomarkers could assist in increasing the index of suspicion for the disease, in determining the duration of hospitalization, the severity of disease and the ultimate prognosis of patients. Biomarkers may also assist in decision-making when discussing treatment options or euthanasia with clients.

Unfortunately, there is a dearth of large, comprehensive, prospective, multi-center studies on CPV infection in dogs. The CPV literature (in common with most of the veterinary literature) is thus replete with small, single-center, geographically-biased and largely retrospective studies with resultant conflicting data on seasonal occurrence, breed predisposition and measures of disease severity. Importantly, the retrospective nature of some studies has several shortcomings such as missing data on outcome (Kalli *et al.* 2010), non-uniform management of cases and varying diagnostic criteriae (Ling *et al.* 2012) and many other unknown and known confounders. These confounders include owner socio-economic status; disparate patient vaccination status; varying canine population kinetics and human population densities; differing CPV strains involved in outbreaks; reporting bias and varying emotional bonds

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between clients and their pets - all of which have significant effects on mortality- and euthanasia rates and other inferences drawn from these studies.

Nevertheless, some converging biomarker trends have emerged from the CPV literature, providing insight into the infinitely complex tapestry of microbe-host interactions. One such trend is the tightly reported mortality rate of approximately 16 - 20%, despite aggressive therapy, in different studies over consecutive years in our institution (Goddard et al. 2008, Schoeman and Herrtage 2008, McClure et al. 2013), that correlate very well with the rates of 21% and 25% reported from the University of Missouri (Mann *et al*. 1998).

Current literature depicts a condition characterized by marked systemic inflammatory response syndrome (SIRS) in the presence of a severely compromised and denuded gut villous surface, impaired white blood cell numbers, signature anti-inflammatory responses and a persistently high mortality rate, despite improvements in therapeutic interventions over the years. This paper is an attempt at discussing different biomarkers that each in their own right depict part of the elephant (Saxe and Galdone 1964), but together ultimately allows a fuller picture to emerge.

Parvoviridae

Parvoviruses are small, non-enveloped, single-stranded DNA viruses that are known to cause disease in a variety of mammalian species. Most parvoviruses are species-specific. Viral replication occurs only in certain rapidly-dividing cells like intestinal crypt epithelial cells, precursor cells in the bone marrow and myocardiocytes, resulting in cell death and loss due to failure of mitosis.

In 1967, parvovirus was first discovered as a cause of gastrointestinal and respiratory disease in dogs and was subsequently called the minute virus of canines, formerly known as CPV-1. In 1978, reports of outbreaks of an unfamiliar contagious enteric disease were reported and the causal agent was isolated as a new species of the family Parvoviridae; subsequently named CPV-2. In the 1980’s a new CPV-2 strain emerged and was designated CPV-2a. The virus quickly mutated again and a new strain, CPV-2b, emerged in 1984 (Parrish et al. 1988). Twelve years ago, a new strain with potentially higher virulence and ability to infect older and vaccinated dogs, CPV-2c, also emerged (Buonavoglia *et al*. 2001). All currently known CPV-2- and CPV-2a-derived virusses are monophyletic, indicating that a single cross-species transmission event gave rise to all CPV strains (Hoelzer *et al*. 2008).

Epidemiology

Acute CPV-2 enteritis can manifest in dogs of any breed, age or sex, but puppies between 6 weeks and 6 months appear to be more susceptible. Low bodyweight was found to be a significant risk factor for death, even after adjusting for age (Dossin *et al*. 2011). In a recent study, the highest mortality rates were seen in dogs less than 6 months of age, yet this parameter failed to achieve significance (Ling *et al*. 2012). Interestingly, the above study also demonstrated higher case fatality rates in summer compared to other

seasons; putatively ascribing it to improved viral particle transmission and increased use of boarding and kennel facilities during the warmer holiday months (Ling *et al.* 2012). Data on the seasonality of CPV enteritis is conflicting, most likely owing to different climatic conditions and other varying risk factors in different countries (McCaw and Hoskins 2006, Kalli *et al*. 2010, Pospischil and Yamaho 1987).

Immunity to CPV following infection or vaccination is long-lived, and therefore the most susceptible animals are puppies born into the population. Certain breeds have been shown to be at increased risk for severe CPV enteritis, including the rottweiler, doberman pinscher, American pit bull terrier, Labrador retriever, German shepherd and Yorkshire terrier (Glickman *et al*. 1985; Pospischil and Yamaho 1987; Houston *et al*. 1996; McCaw and Hoskins 2006). A recent Australian study demonstrated higher case fatality rates in hounds, gundogs and non-sporting pedigree groups than in mixed breed dogs (Ling *et al*. 2012). A Greek study broadly supported these findings by demonstrating increased risk of CPV infection in purebred rather than in mixed-breed puppies, but failed to demonstrate any particular breed predisposition (Kalli *et al*. 2010). It is not yet established whether the above trends are due to differences in immune competence between specific breeds or breed-specific vaccine unresponsiveness or sheer breed popularity in some studies.

CPV-2 spreads rapidly among dogs via the faecal-oral route (direct transmission) or through oro-nasal exposure to fomites contaminated by faeces (indirect transmission). Marked plasma viraemia is observed 1 to 5 days after infection and clinical signs occur after an incubation period of 3-7 days (Decaro and Buonavoglia 2012). Parvovirus infects the germinal epithelium of the intestinal crypts, causing epithelial destruction and villous collapse. As a result, normal cell turnover (usually 1 to 3 days in the small intestine) is impaired, leading to the characteristic pathologic lesion of shortened and atrophic villi (Macartney *et al*. 1984). During this period of villous atrophy the small intestine loses its absorptive capacity. The extensive lymphocytolysis in the thymic cortex, compared to other lymphoid tissues, mirrors the high mitotic rate found in this organ. It is thus not surprising that infected puppies develop severe lymphopaenia (Goddard and Leisewitz 2010).

Clinical manifestations

Enteritis and myocarditis were the two disease entities initially described with CPV-2 infection, but myocarditis is very rarely seen of late. Hence, acute enteritis is the current most common manifestation of the disease with non-specific initial clinical signs such as anorexia, depression, lethargy and fever. Subsequent, more specific clinical signs include vomiting and small bowel diarrhoea that can range from mucoid to haemorrhagic. Due to large fluid and protein losses through the gastrointestinal tract, dehydration and hypovolaemic shock develop rapidly. Marked abdominal pain is a feature of CPV enteritis and can be due to either acute gastroenteritis or less commonly, intestinal intussusception.

Intestinal tract damage secondary to viral infection increases the risk of bacterial translocation and subsequent coliform septicaemia.

This may lead to the development of a systemic inflammatory response that can progress to septic shock and ultimate death. *Escherichia coli* has been recovered from the lungs and liver of infected puppies. Pulmonary lesions similar to those found in humans with adult respiratory distress syndrome have been described (Turk *et al*. 1990; Otto *et al*. 1997). Endotoxin and tumor necrosis factor are present in measurable quantities in the blood of infected puppies and a significant association exists between rising TNF activity and mortality (Yilmaz and Senturk 2000; Otto *et al*. 2000). Endotoxin and pro-inflammatory cytokines are potent mediators of the systemic inflammatory response and activators of the coagulation cascade. The odds of death were also higher in puppies that met the criteria for SIRS (heart rate > 140 beats/min, respiratory rate > 30 breaths/min, temperature > 39.2^0C or < 37.8^0C) than for those that did not (Kalli *et al*. 2010).

Typical ultrasonographic changes that are considered indicative of CPV enteritis include fluid-filled, atonic small and large intestines in common with clinical findings on abdominal palpation. There is duodenal and jejunal mucosal layer thinning with or without indistinct wall layers and irregular luminal-mucosal surfaces; extensive duodenal and/or jejunal hyperechoic mucosal speckling; and duodenal and/or jejunal corrugations. The extensive intestinal lesions correlate with the histopathological findings of villous sloughing, mucosal erosion and ulceration, and crypt necrosis, without any sonographically detectable lymphadenopathy (Stander *et al*. 2010). Additionally, the severity of the sonographic changes is positively correlated with the clinical severity of the patients (Stander *et al*. 2010).

Growing evidence supports the use of early enteral nutrition. Puppies receiving early enteral nutrition via a nasoesophageal tube, compared to puppies that received nil per os until vomiting cease, show earlier clinical improvement, significant weight gain, as well as improved gut barrier function, which could limit bacterial or endotoxin translocation (Mohr *et al*. 2003).

Clinicopathologic biomarkers

Haematology

The leukocyte count during CPV enteritis is generally significantly depressed with a transient lymphopaenia being the most consistent finding (Macartney *et al*. 1984; Brunner and Swango 1985; Goddard *et al*. 2008; Ling *et al*. 2012). Non-survivors have significantly lower total leucocyte, neutrophil, band neutrophil, lymphocyte and eosinophil counts than survivors at admission to hospital (O'Sullivan *et al*. 1984; Mason *et al.* 1987; Dossin *et al*. 2011; Yilmaz and Senturk 2007). The haematological changes are attributable to destruction of haematopoietic progenitor cells. This results in a short supply of leukocytes (specifically neutrophils) to the markedly inflamed gastrointestinal tract. A lack of cytopaenia, specifically normal total leukocyte and lymphocyte counts, had a positive predictive value of 100% for survival 24 hours post-admission. The puppies that were destined for recovery

had a rebound increase in the lymphocyte count 24 hours after admission (Potgieter *et al*. 1981; Goddard *et al*. 2008).

A marked depletion of the granulocytic, erythroid and megakaryocytic cell lines in the bone marrow is present, followed by hyperplasia of the granulocytic and erythroid elements during convalescence. These changes probably reflect the effect of endotoxaemia (Potgieter *et al*. 1981; Boosinger *et al*. 1982). Despite the severe changes seen in the blood precursor cell lines, it appears that early pluripotent cells are spared. Increased plasma granulocyte colony-stimulating factor (G-CSF) concentration is observed in CPV enteritis just after the onset of neutropenia, which then decreases to undetectable levels once the neutropenia has resolved (Cohn *et al.* 1999). Anaemia is a fairly common haematological finding in CPV enteritis, especially in the later phases of severe disease due to a combination of intestinal haemorrhage and rehydration therapy (Jacobs *et al*. 1980). Increased levels of lipid peroxides in erythrocytes and an alteration in antioxidant enzyme concentrations, indicating a state of oxidative stress, may also play a role in anaemia pathogenesis in these patients (Panda *et al*. 2009).

Coagulation abnormalities

Evidence of hypercoagulability without disseminated intravascular coagulopathy has been documented in puppies with CPV enteritis. It is thought to be due to an endotoxin- or cytokine-mediated procoagulant effect on endothelial cells. Loss of antithrombin (AT) through the gastrointestinal tract, as well as consumption of AT as a result of endotoxin-mediated activation of coagulation, and hyperfibrinogenaemia can contribute to the hypercoagulable state seen in CPV enteritis (Otto *et al*. 2000).

Serum biochemistry changes

Infection-induced serum biochemistry abnormalities are non-specific. Severe hypokalemia due to anorexia, vomiting, and diarrhoea may contribute to depression and weakness. Other electrolyte abnormalities (i.e. hyponatraemia and hypochloraemia) may also occur secondary to vomiting and diarrhoea (Heald *et al*. 1986, Nappert *et al*. 2002). Hypoalbuminemia may contribute to reduced total blood calcium concentrations (Jacobs *et al.* 1980).

Although total magnesium concentration has been found to be a prognostic indicator in critically ill humans, total as well as ionized magnesium concentrations were not associated with outcome in CPV enteritis (Mann *et al*. 1998). Serum electrophoresis profiles have shown relative and absolute hypoalbuminaemia, hypogammaglobulinaemia and hyperalpha-2-globulinaemia. The decrease in plasma proteins throughout the course of the disease is most likely due to a combination of a protein-losing enteropathy, intestinal haemorrhage, SIRS-mediated vascular permeability and subsequent rehydration therapy. The increase in alpha-2 globulins is most likely due to the hepatic synthesis of acute phase proteins (APP) stimulated by leukocyte endogenous mediators that are associated with tissue damage and inflammation (van den Broek 1990). Acute phase protein generation occurs at the expense of albumin generation in critical illness.

High serum C-reactive protein (CRP) levels at admission, 12- and 24 hours post admission are positively associated with mortality (Kocaturk *et al*. 2010; McClure *et al*. 2013). Elevated blood urea, creatinine and inorganic phosphate are associated with dehydration. Elevation in liver enzymes may occur as a result of hepatic hypoxia secondary to severe hypovolaemia or the absorption of toxic substances due to loss of the gut barrier (Jacobs *et al*. 1980).

Lipopolysaccharide (LPS) or endotoxin is released from Gram-negative bacteria and is a putative trigger for the host response known as sepsis (Amersfoorth *et al*. 2003). In addition to their roles in cholesterol and lipid transport, plasma lipoproteins bind the bioactive portion of the endotoxin molecule, preventing it from stimulating monocytes, macrophages and other LPS-responsive cells, thereby providing an important host mechanism for controlling responses to endotoxin (Kitchens *et al*. 2003). Corroborative data in CPV-infected dogs showed decreased serum total cholesterol and high-density lipoprotein cholesterol levels, but increased serum triglyceride levels and asserted that hypocholesterolemia may be used as a marker of the severity of CPV enteritis (Yilmaz and Senturk 2007).

Erythrocytic oxidative stress indices, as evidenced by increased lipid peroxide levels are significantly elevated in CPV infected dogs. These dogs also have a concomitant decrease in serum zinc concentrations, which is explained by the fact that zinc is a micronutrient that forms an essential part of the anti-oxidant superoxide dismutase enzyme system (Panda *et al*. 2009).

Studies on acid-base status in CPV enteritis show that most puppies have normal blood pH, some are alkalaemic and a small minority is acidaemic, depending on the severity of the vomiting (i.e. loss of hydrogen and chloride ions) or the origin of the diarrhoea (i.e. small versus large intestine) (Heald *et al*. 1986). The majority of cases that has normal blood pH, show high-normal range pH, despite low plasma HCO_3^- and CO_2^-, which indicate compensated metabolic acidosis probably due to excessive loss of HCO_3^- through the intestinal tract and compensatory tachypnoea (Nappert *et al*. 2002). The metabolic acidosis seen in CPV enteritis is however readily corrected and is not exacerbated by D-lactate production by the bacterial population within the large intestine (Nappert *et al*. 2002). In a study that compared the traditional Henderson Hasselbach and Stewart strong ion difference (SID) models to evaluate the metabolic component of the acid base disturbances seen in CPV, it was shown that the disturbance typical for CPV was a SID acidosis with a concomitant non-volitile weak acid (chiefly albumin) alkalosis. Changes in electrolytes (especially chloride) were determined to be the most important factor in the acid-base disturbances in CPV (Burchell e*t al*.2013 manuscript submitted).

Plasma citrulline concentration is a reliable marker of global enterocyte mass in humans and is markedly decreased in diffuse small intestinal diseases. CPV enteritis is associated with a severe (93%)decrease in plasma citrulline concentrations compared to normal dogs, but no significant difference is noted between survivors and

non-survivors (Dossin *et al*. 2011). This marked decrease of citrulline in CPV enteritis corroborates the similarity between CPV-induced pathology and acute human villous atrophy-associated diseases - a set of diseases that induces the most severe depletion of citrulline. However, human sepsis and critical illness have also been implicated as causing hypocitrullinaemia and both factors are also present in CPV enteritis in addition to the primary intestinal damage (Otto *et al*. 1997; Schoeman and Herrtage 2008). To what extent hypocitrullinaemia and the resultant bacterial translocation across a denuded villous surface contributes to sepsis in CPV enteritis, as has been documented in humans undergoing myeloablative treatment, remains to be discovered (Herbers *et al*. 2008).

Endocrine biomarkers

The response of the adrenal- and thyroid gland to critical illness is essential for survival. Similar to critical illness in humans, high serum cortisol and low serum thyroxine (T4) concentrations at 24 and 48 hours after admission are associated with mortality in CPV infection (Schoeman *et al*. 2007a; Schoeman and Herrtage 2008). Cortisol is the signature hormone involved in the body's response to inflammation through the hypothalamic-pituitary adrenal axis. The high cortisol concentration and its positive correlation with disease severity thus support the marked inflammatory nature of CPV infection and, in turn, the positive correlation of SIRS with mortality in this disease (Kalli et al. 2010). Both high cortisol and high CRP concentrations and their positive correlation with mortality in several studies on CPV infection and canine babesiosis further support the role of inflammation and the body's resultant anti-inflammatory response as markers of disease severity (Schoeman *et al*. 2007a; Schoeman *et al*. 2007b; Kocaturk *et al*. 2010, Koster *et al*. 2011; McClure *et al*. 2013).

The use of biomarkers to guide diagnosis and decision-making.

Taken together, it would seem that a number of biomarkers (objective measures in their broadest sense) are useful in increasing the index of suspicion of a diagnosis of CPV infection and another set are useful in predicting outcome. The authors fully acknowledge that many of these biomarkers are not bedside tests, but they are summarized below for illustrative purposes to summarize current knowledge on prognostic biomarkers in CPV infection. Additionally, cut-off values described in the literature are fraught with limitations and would translate poorly across geographical differences and the myriad of attendant factors that are unique to each patient and/or laboratory.

Furthermore, a critical appraisal of the literature reveals that very few of these prognostic biomarkers have undergone multivariate analysis in the form of logistic regression or Cox proportional hazards analysis. These statistical analyses are more robust and result in odds ratios or relative risk of mortality, enabling readers to quantify the risk associated with a specific factor or set of factors. Instead, most prognostic biomarkers associated with CPV infection have resulted from simple univariate statistics that demonstrated significant differences between dogs that died and

those that survived. Readers are referred to a very interesting review paper highlighting the criteriae that should be satisfied by a study evaluating the predictive ability of a test (Levine et al. 1991).

In sum, and in spite of the shortcomings cited above, a purebred puppy between the age of 6 weeks and 6 months presenting with vomiting and mucoid to haemorrhagic diarrhoea, that has leucopaenia and marked hypocitrullinaemia has CPV infection until proven otherwise.

Furthermore, a number of studies have suggested that the optimal time to prognosticate in CPV enteritis is at 24 hours post admission and not at the time of admission. A patient is likely to have a poor prognosis if it is purebred, has a low bodyweight and, after 24 hours of intensive therapy the following biomarker levels are present: Severe persistent leuko- and lymphopaenia, a persistently elevated or rising serum cortisol concentration (>224 nmol/l), severe hypothyroxinaemia (<2.8 nmol/l), hypocholesterolaemia (< 2.6 mg/dl) and persistently elevated serum CRP (> 97.3 mg/l) and/or TNF concentrations (Table 1).

Conversely, the literature would suggest that puppies with a good prognosis are those that are of mixed breed, > 6 months old and show the following biomarker values: total leucocyte count > 4.5 x 10^3/uL, lymphocyte count > 1 x 10^3/uL and mature neutrophil count > 3 x 10^3/uL, all associated with a 100% survival when measured at 24 hours post admission (Goddard *et al.* 2008); a serum cortisol < 224 nmol/l associated with a 96% survival when measured at 48 hours after admission (Schoeman *et al*. 2007a) and a serum thyroxine concentration > 2.8 nmol/l associated with 100% survival when measured at 24 hours after admission (Schoeman *et al*. 2007a) as well as a HDL-cholesterol of > 1.3 mmol/l associated with a 100% survival when measured at admission.

Last, but not least, the availability of prognostic tests does not relieve the clinician from the burden of making clinical decisions. The clinician dealing with the CPV enteritis patient must decide which prognostic test to measure and what particular threshold value would constitute enough evidence to support a decision for euthanasia or continued treatment. Such judgements are commonplace in clinical medicine where clinicians must weigh the best available evidence for their particular socio-economic situation and exert a value judgement on the cost-benefit ratio of the ensuing decision. Clinicians would be gratified to learn that the authors prefer to use a composite clinical score comprising parameters of severity for vomiting, diarrhoea, capillary refill time, mucous membrane assessment and general demeanour as an indispensible prognosticating tool (Mohr *et al*. 2003), supplemented by the judicious use of affordable and readily do-able biomarkers such as haematology and bedside endocrine tests.

Table 1

Prognostic biomarkers of disease severity in CPV enteritis, indicative of a poor prognosis.

Biomarker category	**Biomarker of poor prognosis**	**Reference**
Historical	Summer presentation	Ling *et al*. 2012
	Vomiting (increased duration of hospitalisation)	Kalli *et al*. 2010
Signalment		
	Younger age	Schoeman and Herrtage 2008
	Purebred dog	Kalli *et al*. 2010
	Low bodyweight	Dossin *et al*. 2011 Schoeman and Herrtage 2008
Haematology		
	Leukopaenia	O'Sullivan et al 1984 Mason et al 1987 Dossin *et al*. 2011, Goddard *et al*. 2008
	Neutropenia	Yilmaz and Senturk 2007 Dossin et al. 2011
	Lymphopaenia	Dossin *et al*. 2011, Goddard *et al*. 2008
Serum biochemistry		
	High serum CRP	Kocaturk *et al*. 2010; McClure *et al*. 2013
	High serum TNF	Yilmaz and Senturk 2000; Otto *et al*. 2000
	Low serum albumin (increased duration of hospitalisation)	Kalli *et al*. 2010
	Low serum cholesterol	Yilmaz and Senturk 2007
Endocrine		
	High serum cortisol	Schoeman *et al*. 2007a
	Low serum thyroxine	Schoeman *et al*. 2007a, Schoeman and Herrtage 2008
Other		
	Positive for SIRS	Kalli *et al*. 2010
	Extensive ultrasonographic intestinal changes	Stander *et al*. 2010

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