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Acute phase response to *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum' infection in FIV-infected and non-FIV-infected cats

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ABSTRACT

The pathogenicity of Haemoplasma spp. in cats varies with '*Candidatus* Mycoplasma haemominutum' (CMhm) causing subclinical infection while *Mycoplasma haemofelis* (Mhf) often induces haemolytic anaemia. The aims of this study were to characterise the acute phase response (APR) of the cat to experimental infection with Mhf or CMhm, and to determine whether chronic feline immunodeficiency virus (FIV) infection influences this response. The acute phase proteins serum amyloid A (SAA), haptoglobin (Hp) and α-1-acid glycoprotein (AGP) concentrations were measured pre-infection and every 7–14 days up to day 100 post-infection (pi) in cats infected with either Mhf or CMhm. Half of each group of cats (6/12) were chronically and subclinically infected with FIV. Marbofloxacin treatment was given on days 16–44 pi to half of the Mhf-infected cats, and on days 49–77 pi to half of the CMhm-infected cats.

FIV-infected animals had significantly lower AGP concentrations, and significantly greater Hp concentrations than non-FIV-infected cats when infected with CMhm and Mhf, respectively. Both CMhm and Mhf infection were associated with significant increases in SAA concentrations, while AGP concentrations were only significantly increased by Mhf infection. Mhf-infected cats had significantly greater SAA concentrations than CMhm-infected animals. Both Mhf and CMhm infections were associated with an APR, with Mhf infection inducing a greater response. Chronic FIV infection appeared to modify the APR, which varied with the infecting Haemoplasma species.

Introduction

Feline haemoplasmas are haemotropic mycoplasmal bacteria. *Mycoplasma haemofelis* (Mhf) is the most pathogenic, often inducing haemolytic anaemia in immunocompetent cats, whilst '*Candidatus* Mycoplasma haemominutum' (CMhm) and '*Candidatus* Mycoplasma turicensis' do not usually cause anaemia unless concurrent disease or immunosuppression is present (Tasker et al., 2009; Tasker, 2010). Subclinical carrier states also exist and the presence of infection does not confirm that haemoplasmosis is the cause of the clinical signs (Tasker, 2010).

The acute phase response (APR) is part of the innate immune response, characterised by changes in serum acute phase protein (APP) concentrations (Cerón et al., 2005), which can be used as diagnostic and therapeutic biomarkers (Cerón et al., 2005; Griebsch et al., 2009; Mitchell et al., 2009). Measurement of APPs is widely used in human medicine (Berbari et al., 2010; Patel et al., 2010; Sage et al., 2010), and there is increasing interest in their use in companion animals (Eckersall, 2010), although there have to date been limited studies carried out in cats (Harvey and Gaskin, 1978; Duthie et al., 1997; Kajikawa et al., 1999; Sasaki et al., 2003; Paltrinieri et al., 2007a,b, 2008; Tamamoto et al., 2008, 2009). To the authors' knowledge, the only study evaluating the APR to haemoplasmosis in cats reported elevated haptoglobin (Hp) concentrations in six animals experimentally infected with Mhf (Harvey and Gaskin, 1978).

The aim of the present study was to characterise the APR to experimental infection with Mhf or CMhm by measuring serum concentrations of three APPs, serum amyloid A (SAA), haptoglobin (Hp) and α-1-acid glycoprotein (AGP). The influence of chronic feline immunodeficiency virus (FIV) infection on the APR to haemoplasma infection was also assessed.

Materials and methods

Study design

This study was performed on serum samples obtained from previous research (Tasker et al., 2006a,b; Fig. 1). All procedures and experiments described were undertaken under a project license approved under the UK Animals (Scientific Procedures) Act 1986. Briefly, these studies had used 24 specific pathogen free (SPF)-derived cats from one of four groups (n = 6 in each case): Groups A and B were

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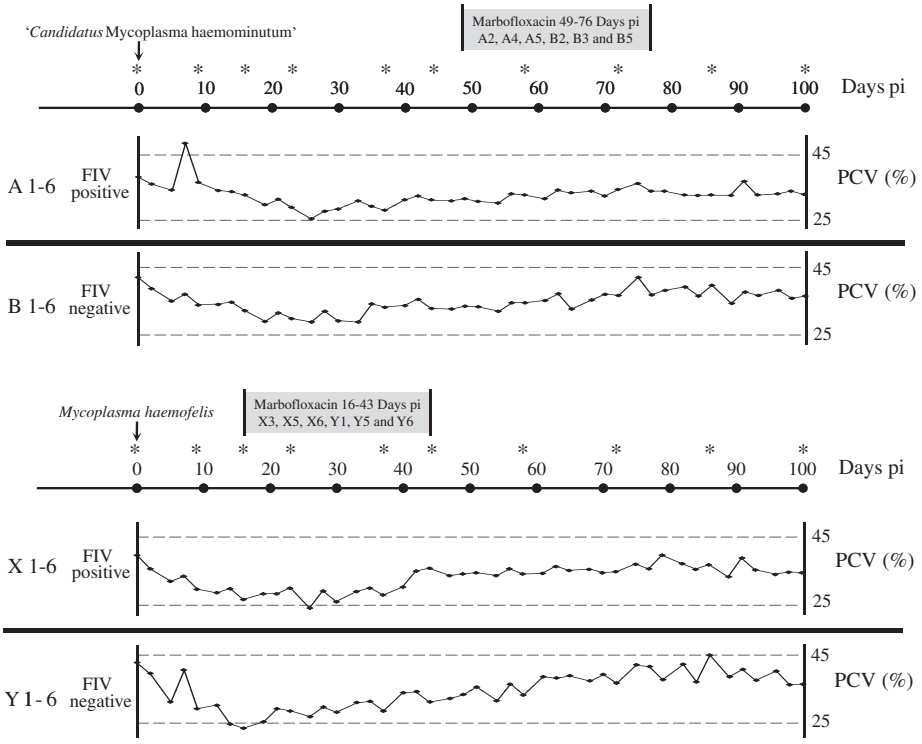

Fig. 1. Schematic illustration of the experimental design for Groups A, B, X and Y. Protocols are indicated against days post-infection (pi). FIV infection status and mean packed cell volume (PCV) (dashed lines indicate reference interval) for each group are indicated. Solid arrow, IV inoculation with Haemoplasma spp.; *sampling times; shaded box, marbofloxacin administration (identity of treated cats shown).

infected with CMhm and Groups X and Y with Mhf, by administering 2 mL of heparinised blood IV from a CMhm- or Mhf-infected donor cat. Groups A and X were chronically infected (23–32 months) with FIV (Glasgow8 strain).

All cats were clinically normal at study commencement. Three cats from each group were randomly selected and given marbofloxacin (Marbocyl, Vétoquinol) at 2 mg/kg orally, once daily for 28 days: between days 49–76 post-infection (pi) for the CMhm-infected cats (cat numbers A2, A4, A5, B2, B3 and B5), and between days 16–43 pi for the Mhf-infected cats (cat numbers X3, X5, X6, Y1, Y5 and Y6) (Tasker et al., 2006a,b). Differences in the timing of this treatment reflected the different time courses of infection: the Mhf-infected cats were treated early in the course of infection when anaemia was present, while the CMhm-infected cats, with subclinical infection, were treated later in the course of infection when CMhm copy numbers remained high.

Each group received the marbofloxacin treatment in order to evaluate the response of each Haemoplasma spp. All cats were given subcutaneous fluid therapy (lactated Ringer's solution) if their PCV fell below 15% and/or the animal exhibited evidence of dehydration. Serum samples were collected pre-infection (day 0), weekly between days 9–44 pi (excluding day 30 pi), and then fortnightly until day 100 pi.

Assays for acute phase proteins

Serum amyloid A concentrations were determined using an immunoturbidimetric method used in humans and validated in cats (Hansen et al., 2006). Alpha-1-acid glycoprotein concentrations were measured using a rapid immunoturbidimetric assay validated for use in cats (Bence et al., 2005). Haptoglobin concentrations were determined using a commercially available kit (Tridelta Development Ltd.). The SAA concentration was measured first, followed by the AGP and Hp concentrations, although inadequate amounts of serum were available to analyse all APPs in all samples: in Group A, seven samples were not analysed for Hp and three were not analysed for AGP; in Group B, four samples were not assessed for Hp or AGP; in Group X, five and two samples were not analysed for Hp and AGP, respectively; and in Group Y, one sample not examined for Hp or AGP.

Statistical analysis

Data were entered into and validated within a database (Excel 2008, Microsoft), and then exported into SPSS software (version 18.0) for further analysis. Significance was set at $P < 0.05$. Statistical analysis was only performed on APP concentrations measured pre-treatment to ensure no confounding effects of treatment. To assess the effect of chronic FIV infection on the APR to haemoplasma infection, Mann–Whitney *U* tests were used to compare each APP concentration pi between the FIV-infected and non-FIV-infected animals, for both CMhm and Mhf. To assess the effect of each infection on the APR, the mean of the APP concentration pi (on days 9, 16, 23, 37 and 44 for CMhm and on days 9 and 16 for Mhf, respectively) was compared with pre-infection (day 0) values using a Wilcoxon-signed ranks test. Conventional two-tailed testing was used to assess changes in Hp and AGP concentrations.

As SAA pre-infection concentrations were below detection limits, the SAA concentration changes pi were investigated using one-tailed testing. These analyses were carried out using non-parametric tests due to the small sample sizes. Two-way repeated measures ANOVA were performed for each APP on days 9–16 pi (the pre-treatment measurements available for both CMhm and Mhf), using FIV and Haemoplasma spp. as grouping variables. Residuals from this analysis tested satisfactorily for normality and homogeneity of variance, confirming the suitability of this test.

Results

Effect of chronic FIV infection on the acute phase response to haemoplasma infection

Pre-existing FIV infection did not significantly affect SAA concentrations following CMhm or Mhf infection, Hp concentrations following CMhm infection, or AGP concentrations following Mhf infection (Table 1). Therefore, the SAA concentration data for CMhm-infected (Groups A and B) and Mhf-infected (Groups X and Y) cats, the Hp data for CMhm-infected (Groups A and B) cats, and the AGP data for Mhf-infected (Groups X and Y) cats, were combined for further analyses.

FIV infection did however affect Hp concentrations following Mhf infection, with FIV-infected cats having significantly greater Hp concentrations than non-FIV-infected animals (Table 1). FIV infection also affected AGP concentrations following CMhm infection, with FIV-infected cats having significantly lower AGP concentrations than non-FIV-infected animals (Table 1). Thus, Hp concentrations in Mhf-infected cats (Groups X and Y), and AGP concentrations in CMhm-infected cats (Groups A and B), were analysed separately.

Table 1
Statistical analysis of data for each infecting Haemoplasma spp. and for each acute phase protein (APP) comparing non-FIV-infected and FIV-infected cats and pre- (day 0) and post haemoplasma-infection, respectively. Reference intervals are given for each APP. Statistically significant results are highlighted in bold, with the median (and range) measurements for each group. For each APP, where no significant difference was found between FIV-infected and non-FIV-infected cats, data were combined for the comparison of pre- and post-haemoplasma-infection data.

Infecting Haemoplasma spp.	Serum amyloid A (⩽18.53 mg/L)[a]		Haptoglobin (⩽3.84 g/L)		α-1-Acid glycoprotein (⩽0.48 g/L)	
	FIV vs. non-FIV infected	Pre- vs. post-infection	FIV vs. non-FIV infected	Pre- vs. post-infection	FIV vs. non-FIV infected	Pre- vs. post-infection
'*Candidatus* Mycoplasma haemominutum'	$z = -0.480$, $P = 0.699$	**$z = -2.982$, $P < 0.001$** Pre-infection ⩽0.38 mg/L (⩽0.38 mg/L) vs. post-infection 0.83 mg/L (⩽0.38–24.02 mg/L)	$z = -0.480$, $P = 0.699$	$z = -1.49$, $P = 0.151$	**$z = -2.562$, $P = 0.009$** FIV 0.08 g/L (0.03–0.13 g/L) vs. non-FIV 0.26 g/L (0.09–0.36 g/L)	FIV: $z = -1.363$, $P = 0.219$ Non-FIV: $z = -0.943$, $P = 0.438$
Mycoplasma haemofelis	$z = -0.480$, $P = 0.699$	**$z = -3.059$, $P < 0.001$** Pre-infection ⩽0.38 mg/L (⩽0.38 mg/L) vs. post-infection 32.2 mg/L (1.45–77 mg/L)	**$z = -2.402$, $P = 0.015$** FIV 2.43 g/L (1.05–3.65 g/L) vs. non-FIV 0.41 g/L (0.15–2.45 g/L)	FIV: $z = -1.782$, $P = 0.094$ Non-FIV: $z = -0.105$, $P = 1.00$	$z = -1.761$, $P = 0.093$	**$z = -2.197$, $P = 0.027$** Pre-infection 0.15 g/L (0–0.75 g/L) vs. post-infection 0.58 g/L (0.06–1.46 g/L)

[a] The lower limit of detection for serum amyloid A was 0.38 mg/L.

Effect of haemoplasma infection on serum amyloid A concentrations

Up to day 44 pi following CMhm infection, 7/12 cats demonstrated rises in SAA concentration (Fig. 2A), with peak concentrations on days 16 or 23 pi. Mean SAA concentrations were significantly elevated up to day 44 pi compared with day 0 (Table 1), but had returned to baseline levels in all but one of the CMhm-infected cats by day 44 pi, just prior to the marbofloxacin treatment. Serum amyloid A concentrations in this cat (B1) had returned to baseline levels by day 58 pi. Increased SAA concentrations were detected in 11/12 Mhf-infected cats (Fig. 2B), with concentrations peaking around day 9 pi. Mean SAA concentrations were significantly elevated up to day 16 pi compared with day 0 (Table 1).

Marbofloxacin treatment was associated with decreased SAA concentration in some Mhf-infected cats (e.g. X6 and Y6), but in others (e.g. X5 and Y1) the concentration of this AAP had started decreasing prior to treatment. A two-way ANOVA did not detect an interaction effect between FIV and haemoplasma infection on SAA concentrations ($F = 3.122$, $P = 0.092$) up to day 16 pi, however Mhf-infected cats had significantly higher mean SAA concentrations (27.05 ± 0.4 mg/L) compared with CMhm-infected animals (0.94 ± 0.4 mg/L) ($F = 31.706$, $P < 0.001$). FIV infection status did not have a significant effect ($F = 0.790$, $P = 0.385$).

Effect of haemoplasma infection on haptoglobin concentration

Variably increased Hp concentrations occurred in 10/12 CMhm-infected cats up to day 23 pi, but mean concentrations were not significantly different up to day 44 pi compared with day 0 values (Fig. 3A; Table 1). By the time marbofloxacin treatment commenced on day 49 pi, Hp concentrations were already declining in 8/12 of the CMhm-infected cats. Mhf-infected animals demonstrated both increased and decreased Hp concentrations from baseline levels on day 9 pi (Fig. 3B and C). Mean Hp concentrations were not significantly different up to day 16 pi in both non-FIV-infected and FIV-infected cats compared with day 0 (Table 1), although a trend for Hp concentrations to decrease following Mhf infection was observed in FIV-infected cats. Haptoglobin concentrations fell from day 16 pi onwards, and by day 23 pi most cats had concentrations below pre-infection levels. Marbofloxacin

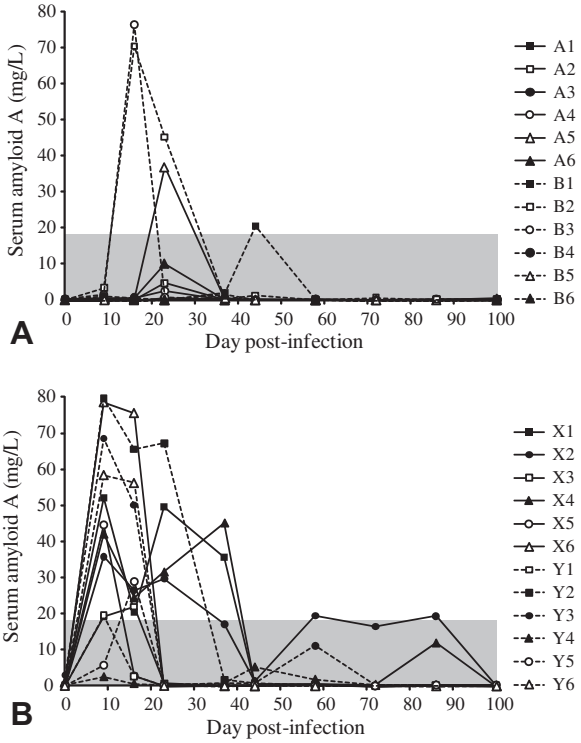

Fig. 2. Graph illustrating alterations in serum amyloid A (SAA) concentrations over time: (A) following '*Candidatus* Mycoplasma haemominutum' (CMhm) infection of FIV-infected (Group A, solid line) and non-FIV-infected (Group B, dashed line) cats. Marbofloxacin was given to a proportion of animals (indicated by open markers) on days 49–77 pi; (B) following *Mycoplasma haemofelis* (Mhf) infection of FIV-infected (Group X, solid line) and non-FIV-infected (Group Y, dashed line) cats. Marbofloxacin was given to a proportion of animals (indicated by open markers) on days 16–44 pi. The shaded panels represent the reference interval for SAA concentration in cats.

treatment did not increase this rate of decline. A two-way ANOVA showed no significant: difference in Hp concentrations between Mhf- and CMhm-infected cats up to day 16 pi ($F = 0.745$, $P = 0.404$); effect of FIV status ($F = 3.56$, $P = 0.082$); effect of interaction between these factors ($F = 3.178$, $P = 0.098$).

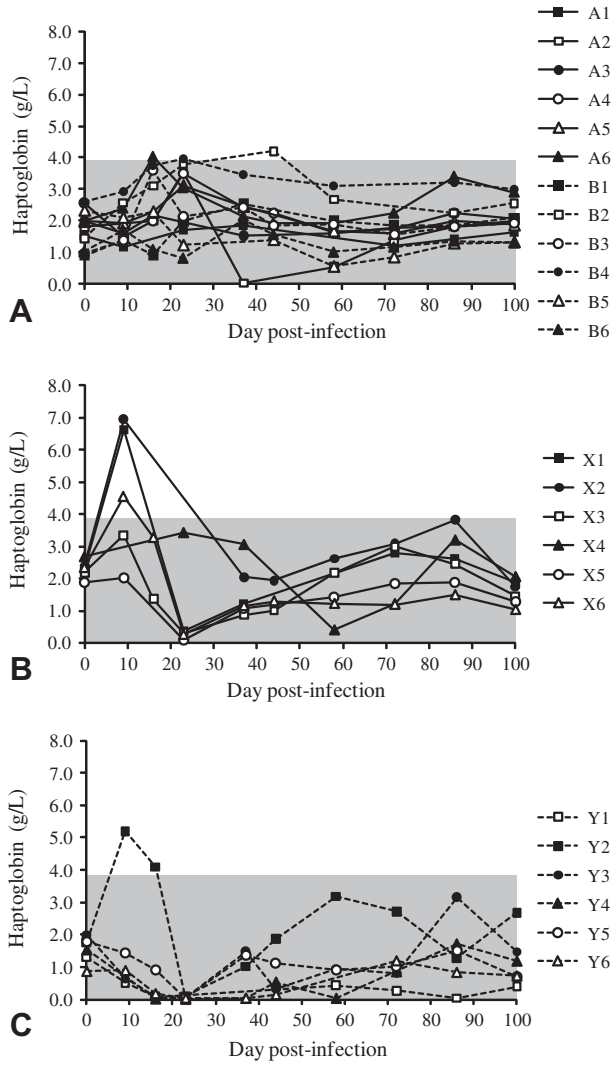

Fig. 3. Graph illustrating alterations in serum haptoglobin (Hp) concentrations over time: (A) following '*Candidatus* Mycoplasma haemominutum' (CMhm) infection of FIV-infected (Group A, solid line) and non-FIV-infected (Group B, dashed line) cats. Marbofloxacin was given to a proportion of animals (indicated by open markers) on days 49–77 pi; (B) following *Mycoplasma haemofelis* (Mhf) infection of FIV-infected cats (Group X, solid line). Marbofloxacin was given to a proportion of animals (indicated by open markers) on days 16–44 pi; (C) following Mhf infection of non-FIV-infected cats (Group Y, dashed line). Marbofloxacin was given to a proportion of animals (indicated by open markers) on days 16–44 pi. The shaded panels represent the reference interval for Hp concentration in cats.

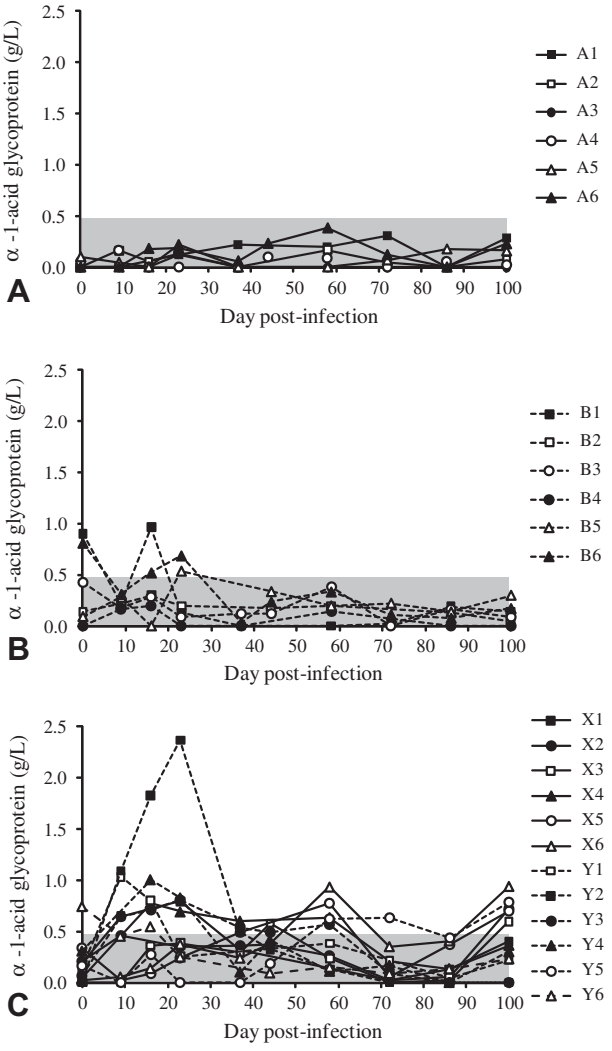

Fig. 4. Graph illustrating alterations in serum α-1-acid glycoprotein (AGP) concentrations over time: (A) following '*Candidatus* Mycoplasma haemominutum' (CMhm) infection of FIV-infected cats (Group A, solid line). Marbofloxacin was given to a proportion of animals (open markers) on days 49–77 pi; (B) following CMhm infection of non-FIV-infected cats (Group B, dashed line). Marbofloxacin was given to a proportion of animals (open markers) on days 49–77 pi; (C) following *Mycoplasma haemofelis* (Mhf) infection of FIV-infected (Group X, solid line) and non-FIV-infected (Group Y, dashed line) cats. Marbofloxacin was given to a proportion of animals (open markers) on days 16–44 pi. The shaded panels represent the reference interval for AGP concentration in cats.

Effect of haemoplasma infection on α-1-acid glycoprotein concentration

Up to day 44 pi, CMhm infection was not associated with significantly different AGP concentrations in either FIV-infected or non-FIV-infected cats compared with day 0 values (Fig. 4A and B; Table 1). Small fluctuations in AGP concentration occurred throughout the study in both treated and untreated CMhm-infected cats, with most variation seen in non-FIV-infected cats up to day 23 pi of CMhm infection. Mhf infection was associated with variable AGP concentrations, with varying peaks and fluctuations evident throughout, and one cat (Y2) developing a marked elevation in the concentration of this AAP at day 23 pi (Fig. 4C). Significantly higher mean AGP concentrations were recorded up to day 16 pi following Mhf infection compared with day 0 (Table 1). A two-way ANOVA did not demonstrate a significant difference in AGP concentrations between Mhf- and CMhm-infected cats up to day 16 pi ($F = 3.809$, $P = 0.07$), although significance was approached, with Mhf-infected cats exhibiting larger AGP concentrations (0.368 ± 0.063 g/L) than their CMhm-infected cohorts (0.170 ± 0.052 g/L). The interaction effect was modelled, but was not found to be significant ($F = 1.84$, $P = 0.195$), although a significant effect of FIV status was detected ($F = 14.86$, $P = 0.002$).

Discussion

To the authors' knowledge, this is the first study to comprehensively evaluate the APR of cats to infection with Haemoplasma spp. Given that the serum analysed was obtained during previous studies investigating haemoplasma/chronic FIV infection (Tasker et al., 2006a,b), a concurrent systemic viral infection could have had a confounding effect on our results. A glycan moiety of AGP has been shown to be modified in cats subclinically and terminally-infected with FIV (Pocacqua et al., 2005). In the present study lower AGP concentrations were found in FIV-infected compared with

non-FIV-infected animals following CMhm infection and higher Hp concentrations occurred in FIV-infected compared with non-FIV-infected cats infected with Mhf. However, while these results were statistically significant, measurements for each group of cats remained within reference intervals and numbers in each group were small. These results are in contrast to those of Duthie et al. (1997), where APPs were measured at a single time-point in six cats with terminal, FIV-associated disease. This study found that 5/6 and 4/6 cats had AGP and Hp concentrations above the reference intervals, respectively.

Marked increases in AGP have been found during the acute phase of viral infection with feline coronavirus (Paltrinieri et al., 2004), although decreases in APPs following viral infection, either acute or chronic, have not been described. In patients with human immunodeficiency virus (HIV) infection, many APPs (e.g. C-reactive protein [CRP], Hp, and fibrinogen) are higher compared with non-HIV-infected controls (Jahoor et al., 1999; Treitinger et al., 2001; Delanghe et al., 2010). Elevations in the concentration of APPs, particularly CRP, are associated with increased mortality and opportunistic disease such as *Pneumocystis jirovecii* pneumonia (Nixon and Landay, 2010; Sage et al., 2010) in AIDS patients. CRP is not part of the feline APR (Cerón et al., 2005) and, to the authors' knowledge, there are no studies comparing the APR between HIV and FIV infection. Serum samples taken pre- and immediately post-FIV infection from the cats in the current study were not available for analysis, and were beyond the scope of this study.

The raised SAA concentration observed following infection with both Haemoplasma spp. was evident in most cats by day 16 pi, as previously described in cats with urinary tract disorders and post-surgery (Kajikawa et al., 1999; Sasaki et al., 2003). SAA concentrations usually returned close to pre-infection values by day 44 pi consistent with a self-limiting APR, and despite sustained circulating haemoplasma copy numbers (Tasker et al., 2006a,b). This finding indicates that an elevated SAA concentration in a haemoplasma-infected cat suggests acute (<44 days post-exposure), rather than chronic, infection. Infection with Mhf was associated with significantly higher SAA concentrations than CMhm infection, consistent with Mhf being more pathogenic (Foley et al., 1998; Westfall et al., 2001; Tasker et al., 2006a, 2009). Haematological variables had been previously assessed in these cats (Tasker et al., 2006a,b) with 10/12 Mhf-infected cats developing regenerative macrocytic, hypochromic haemolytic anaemia by day 14 pi.

The rapid increases in SAA observed in these animals by day 9 pi, therefore mirrored the development of anaemia. Although Mhf infection was associated with significantly greater SAA concentrations than infection with CMhm, the latter was also associated with significant increases in SAA, confirming induction of an APR by CMhm, albeit of lower magnitude. Indeed, Tasker et al. (2006a) found that CMhm infection resulted in decreased PCV in all cats, but in only one-third of animals did this decrease to the point of anaemia. The concentration of SAA also correlates with disease severity in feline pancreatitis (Tamamoto et al., 2009), where decreases mirror clinical improvement. In the current study, the fact that treatment was not instigated when the APR was maximal and/or clinical signs were apparent may explain the lack of association between SAA concentrations and treatment found.

Haptoglobin concentrations did not change significantly following either CMhm or Mhf infection, nor was there a significant difference in the concentration of this biomarker between CMhm and Mhf infections. A trend for Hp concentrations to decrease following Mhf infection was seen among the FIV-infected cats, perhaps because circulating Hp complexes with haemoglobin (Hershko et al., 1973), causing transient decreases in Hp during haemolysis, as previously reported in experimentally infected cats (Harvey and Gaskin, 1978). The reason why this was only identified in FIV-infected cats remains unknown, and FIV infection was not associated with more severe haemolysis following intercurrent Mhf infection (Tasker et al., 2006b).

The concentration of AGP was significantly elevated, albeit to a highly variable degree between individuals, during acute Mhf, but not during acute CMhm, infection, possibly due to the more pathogenic nature of Mhf. Thus, AGP may be a less sensitive indicator of the feline APR than SAA, since infection with both Mhf and CMhm was associated with significant increases in SAA. It is likely that particular APPs have different sensitivities in responding to different disease processes, as is the case with SAA and C-reactive protein in humans with kidney allograft rejections (Casl et al., 1995). Although, to the authors' knowledge, a comparison of the sensitivities of SAA and AGP in the feline APR has not been carried out, SAA increases and peaks earlier than AGP and Hp following experimentally-induced inflammation (Kajikawa et al., 1999).

The retrospective nature of the current study resulted in a number of limitations: serum sample volumes were not sufficient to facilitate the analysis of all three APPs in all samples; statistical power and the elimination of variations in individual responses was limited by the relatively small number of animals in each group (this number would have been further reduced if treated and untreated cats had been compared) and treatment periods for the Mhf- and CMhm-infected cats were different, which limited statistical analysis of differences between the two infecting Haemoplasma spp. More frequent sampling during acute infection when the APR is maximal (e.g. daily up to day 30 pi), may have provided greater statistical power by facilitating daily statistical comparisons (Kajikawa et al., 1999). Instead, because there were few data points recorded within this period, statistical analysis was performed on the mean APP concentrations pi for each infecting haemoplasma. Finally, the APR in cats with naturally occurring FIV and Haemoplasma spp. infection may be different from our experimentally infected animals. Differences in disease transmission and/or exposure to other pathogens may be important in this context.

Although not all data were analysed statistically, the present study still provides valuable information as to the long-term changes in APPs following haemoplasma infection and antibiotic treatment.

Conclusions

This study has demonstrated that both Mhf and CMhm, despite differing pathogenicity, are associated with an APR in infected cats. Although APPs are non-specific indicators of inflammation and infection, their measurement may facilitate the differentiation of acute/clinical from chronic/subclinical haemoplasma infection. SAA was found to have particular promise in this context by rising higher following Mhf than CMhm infection, and by rapidly returning to baseline levels after the acute phase of infection.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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