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Whole blood cytokine profiles in cats infected by feline coronavirus and healthy non-FCoV infected specific pathogen-free cats

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In this study, the cytokine profiles of clinically healthy cats naturally infected with feline coronavirus (FCoV), of cats with feline infectious peritonitis (FIP) and of specific pathogen-free (SPF) cats were investigated in whole blood using a traditional reverse-transcriptase polymerase chain reaction (RT-PCR) assay and a semi-quantitative method of analysis based on computerised quantification of positive bands. The low inter-assay coefficient of variation recorded demonstrated that this method is highly repeatable. Compared with SPF cats, cytokine production was upregulated in most of the samples from FCoV-positive non-symptomatic cats. The appearance of a case of FIP in the cattery was associated with an increased expression of cytokines, in particular there was an increased production of IL-1 β and IFN- γ , suggesting that these cytokines might protect infected cats from the disease. This hypothesis was also supported by the low levels of IFN- γ recorded in blood from cats with FIP.

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Cytokines play a central role in the regulation of cell differentiation, proliferation and cell–cell communication (Balkwill and Burke 1989) and differences in their expression are associated with disease activity in immuno-mediated and inflammatory disorders (Mosmann and Sad 1996). The characterisation of cytokine profiles by peripheral blood cells should make it possible to estimate a ‘peripheral immune state’ in both physiological and pathological conditions (Stordeur et al 2002).

Studies on cytokine profiles in cats with feline infectious peritonitis (FIP), a fatal disease induced by feline coronaviruses (FCoVs) provided controversial results (Goitsuka et al 1990, Gunn-Moore et al 1998, Dean et al 2003, Kiss et al 2004), and no information is available about cytokine profiles of non-symptomatic naturally FCoV-infected cats, which are known to have clinicopathological changes consistent with a cytokine-induced protective immunity (Paltrinieri et al 2003,

Giordano et al 2004). The majority of these studies were performed on tissue homogenates or on purified, isolated cell populations like peripheral blood mononuclear cells (PBMCs) (Leutenegger et al 1999, Kipar et al 2001). However, this approach provides only limited information about cell interactions that, in vivo, define cytokine production. Furthermore, some authors have demonstrated that cell separation procedures can influence the mRNA levels of cytokines: the isolation of PBMC by density gradient separation increases the expression of mRNAs for IL-2, IL-4 and TNF- α (Härtel et al 2001), while IFN- γ is higher in whole blood compared to PBMCs from the same individual (Stordeur et al 2002). The use of whole blood can be thus considered an alternative method to evaluate cytokine profiles. This approach reduces both the time and costs associated with cell isolation and purification, requires minimal sample manipulation, and, above all, the data obtained reflect more accurately in vivo events. Whole blood cultures have been extensively used in human medicine (Ham et al 1991, Allen et al 1992), but rarely in veterinary medicine (Yancy et al 2001, Duvigneau et al 2003). To our knowledge, there are no studies

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about evaluation of cytokine expression directly on feline whole blood.

The aims of the present report were: (1) to develop a reverse-transcriptase polymerase chain reaction (RT-PCR) assay to evaluate cytokine profiles in whole blood; (2) to validate a semi-quantitative method for interpretation of RT-PCR results; (3) to investigate the cytokine profiles in whole blood from clinically healthy cats naturally infected by FCoV and from cats with FIP compared to healthy, non-FCoV infected specific pathogen-free (SPF) cats.

Material and methods

Animals and experimental design

This study was performed on 24 blood samples collected from 10 cats grouped as follows (Table 1):

Group 1: Four clinically healthy Persian cats living in an FCoV-endemic cattery of approximately 40 cats. The cattery was managed in order to provide physical separation of cats with specific problems (pregnancies, parturitions, infectious diseases, etc) while asymptomatic cats shared the same environment. Sporadic cases of FIP (1–4 per year) occurred in the cattery in past years, suggesting that the cattery might be considered endemically infected by FCoV. As in most cats living in this environment the colonisation of intestine by FCoVs may be cyclic (Addie and Jarrett 2001), these cats were sampled at intervals ranging from 1 to 3 months in order to assess the possible relationship between the cytokine pattern and the actual FCoV status. Specifically, five samples (T1–T5) were collected

from cats A and D and four samples were collected from cats B (which was not sampled at T4) and C (which was not sampled at T3). The intervals between samples were 90 days from T1 to T2, 35 days from T2 to T3 and from T3 to T4 and 70 days from T4 to T5. Cases of FIP occurred 12 days after sampling 3 and 30 days after sampling 5.

Group 2: Three cats affected by FIP. One of them came from a Maine Coon breeding cattery, the others were two domestic shorthairs living in multi-cat households on which cases of FIP were recorded in the past. All these cats showed clinical signs consistent with FIP. They were sampled just before euthanasia, and then they were submitted for necropsy. In all these three cases, the clinical suspicion of FIP was confirmed by post-mortem examinations that included necropsy, histology and immunohistochemistry for FCoV-antigens, performed as already described (Paltrinieri et al 2003).

Group 3: Three SPF cats permanently kept at the animal facility of the Vetsuisse Faculty of the University of Zurich. At the time of sampling these cats were clinically healthy and did not show any haematological or biochemical abnormalities.

At each sampling, the cytokine pattern in whole blood was investigated and the FCoV status of the cats was assessed by anti-FCoV serology and by RT-PCR for detecting faecal shedding of FCoVs.

Faecal samples were collected from each cat and frozen at -30°C until analysis. Blood was collected from the jugular vein and put into EDTA-coated tubes. A complete blood cell count

Table 1. Breed, sex and age of the examined cats

| Group | Cat | Breed | Sex | Age |
|----------|-----|--------------------|-----|----------|
| (1) FCoV | A | Persian | M | 7 years |
| | B | Persian | M | 5 years |
| | C | Persian | F | 9 years |
| | D | Persian | M | 12 years |
| (2) FIP | E | Domestic shorthair | F | 7 months |
| | F | Maine Coon | F | 1 year |
| | G | Domestic shorthair | F | 8 months |
| (3) SPF | H | Domestic shorthair | M | 10 years |
| | I | Domestic shorthair | M | 1 year |
| | L | Domestic shorthair | M | 1 year |

and a differential leukocyte count were performed on anticoagulated blood using an automated cell counter (Hemat 8, SEAC) and microscopic examination of May Grunwald–Giemsa stained smears, respectively. Blood was then centrifuged to obtain plasma for serology.

RNA extraction and RT-PCR for cytokines

All the samples used for RNA extraction were refrigerated and processed 8 h after sampling. Based on haematological results, we used a volume of whole blood containing a total amount of 3×10^6 white blood cells for RNA extraction. RNA was extracted using a commercially available kit (QIAamp RNA Blood Mini Kit, Qiagen, Milan, Italy) in accordance with the manufacturer's instructions. To avoid contamination by genomic DNA (gDNA), a DNase treatment was performed on-column (RNase-Free DNase, Qiagen). Prior to cDNA synthesis, 1 μ l of RNA from each sample was subjected to PCR analysis for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Samples that demonstrated a PCR product for GAPDH were considered to have

genomic contamination and were subjected to a second DNase treatment (DNA-FREE, Ambion, Cambridgeshire, United Kingdom) and rechecked for DNA contamination. All samples were negative for gDNA after the second DNase treatment. The synthesis of first-strand cDNA templates with oligodT (200 ng/ μ l) was carried out with a commercial kit (Ready-To-Go You-Prime First-Strand Beads, Amersham Biosciences, Piscataway, NJ, USA) in a volume of 33 μ l at 42 °C for 50 min followed by inactivation of reverse transcriptase at 70 °C for 15 min. Samples were stored at –80 °C.

The signal for feline GAPDH on cDNA was used to normalise differences in RNA extraction and variability in RT and PCR efficiencies to allow comparison between samples. In particular, serial dilutions of cDNA from each sample were performed (no dilution, 1:10, 1:100, 1:200, 1:400) and used to carry out a PCR for GAPDH. Primers for GAPDH amplification were designed based on the published sequence (GenBank accession number AB038241) (Table 2). The reaction mix was prepared in a volume of 10 μ l containing 1 μ l of cDNA, 1 \times buffer, 1.5 mM

Table 2. List of the primers and thermal cycling protocols used in the different RT-PCR cytokine assays

| Cytokine | Primer sequence | Product size (bp) | Annealing temperature (°C) | Number of cycles |
|-----------------------------------|---|-------------------|----------------------------|------------------|
| IL-1 β * | Forward: AGTACCTGAACTCACCAGTG Reverse: TAGTCCTGTGACTGTATGGC | 358 | 59 | 35 |
| IL-4 [†] | Forward: GCATTTACCAGCACCTTCG Reverse: GATCGCTTTTAGCCTTTCC | 330 | 55 | 35 |
| IL-6 [†] | Forward: TGCCTGACAAGAATCACTACT Reverse: GAACTACAGCAATCTTAGATG | 333 | 55 | 35 |
| IL-10 [‡] | Forward: CTGCACCCACTTCTCAGTCAGC Reverse: CCACCACCTTGCTCTTGTTT | 332 | 59 | 35 |
| IL-12 _{p40} [‡] | Forward: CAAAGAATTTGCAGATGCTGG Reverse: ATGTCCCTGATGAAGAAGC | 462 | 55 | 35 |
| TNF- α [§] | Forward: CTGCAACTAATCAACCCTC Reverse: TCAGCGCTGAGTCGATC | 423 | 57 | 35 |
| IFN- γ | Forward: TGGTGGGTCGCTTTTCGTAG Reverse: GAAGGAGACAATTTGGCTTTGAA | 85 | 59 | 38 |
| GAPDH | Forward: TCTTCCAGGAGCGAGATCC Reverse: AGGGATGATGTTCTGGCAGC | 398 | 55 | 35 |
| P205 [¶] | GGCAACCCGATGTTTAAACTGG | 223 | 62 | 40 |
| P211 [¶] | CACTAGATCCAGACGTTAGCTC | | | |

*DeLaurier et al 2002.

[†]Gunn-Moore et al 1998.

[‡]Harley et al 1999.

[§]Rottman et al 1995.

^{||}Leutenegger et al 1999.

[¶]Herrewegh et al 1995.

MgCl₂, 0.2 mM of each dNTP, 1 µM of each primer, 0.5 U of Taq Polymerase (Eppendorf) and nuclease-free water. Negative controls, without cDNA, were incorporated with each set of PCR reaction. The PCR reactions were performed in an Eppendorf Mastercycler. The PCR protocol consisted of denaturation at 94 °C (45 s), annealing for 50 s at 55 °C and extension at 72 °C (80 s) for 35 cycles with a final extension step at 72 °C for 10 min. Ten microlitres of reaction product were visualised on a 1.5% agarose gel containing ethidium bromide and an image of the gel was digitally photographed on an ultraviolet transilluminator.

Gel images were then subjected to quantisation of band intensity using a free-download dedicated software (Quantity One Basic Software, Bio-Rad, Hercules, CA, USA). The mean band intensity was calculated after background subtraction. For each sample, we identified the dilutions that showed a comparable intensity (coefficient of variation within each run of less than 10%), which were then used to perform PCR for all cytokines. Thus, we ensured comparable amounts of starting cDNA material were used and minimised the possibility that differences in cytokine levels were due to methodological variability.

The cytokine primer sequences used for feline IL-1β, IL-4, IL-6, IL-10, IL-12_{p40}, TNF-α and IFN-γ were designed by others (Table 2). The optimised PCR protocol for detecting cytokines cDNA was: denaturation at 94 °C (45 s), annealing for 50 s at various temperatures and number of cycles (Table 2) and extension at 72 °C (80 s) with a final extension step at 72 °C of 10 min.

PCR products were identified using a 100 bp standard marker (Gene Ruler 100 bp DNA Ladder plus, Fermentas UAB, Vilnius, LIT). Each primer pair was shown to produce a single PCR product of the predicted size. The gel images of each reaction were analysed as described for GAPDH mRNA analysis.

PCR for FCoV on faeces

Frozen faeces were thawed, then 1 g of each faecal sample was homogenised in 6 ml of saline solution and centrifuged (4000 rpm × 45 min). The supernatants were removed and filtered using a pore size of 0.22 µm (Millipore, Billerica, MA, USA). Three millilitres of each filtered supernatant was applied to a Centricon YM-30 column (Amersham Biosciences) in order to concentrate viral particles to a final volume of 140 µl.

Viral RNA was then extracted using the QIAamp Viral RNA mini Kit (Qiagen) and reverse-transcribed using the Ready-To-Go You-Prime First-Strand Beads kit (Amersham Biosciences), and the FCoV specific primers p211 (20 pmol/ml) (Herrewegh et al 1995). cDNAs were then amplified by PCR using a reaction mix composed of 1 µl of cDNA, 1× buffer, 2 mM MgCl₂, 0.2 mM of each dNTP, 0.25 U of Taq Polymerase (Eppendorf), and 1 µM of each primer: the primers p211 and p205 were used (Herrewegh et al 1995). The thermal cycling protocol used was: 94 °C (2 min), 40 cycles of denaturation at 94 °C (45 s), annealing at 62 °C (40 s), elongation at 72 °C (40 s) and a final elongation step at 72 °C (10 min). In each batch of test, negative controls (reaction mix without cDNA) and positive controls (FCoV cDNA amplified from the vaccine Primucell FIP, Pfizer Animal Health) were included. Amplicons were then run on a 2% agarose gel and visualised using an UV transilluminator. Based on the presence or absence of amplicons, cats were classified as positive or negative.

Serology

All the cats included in the study, including the SPF cats, were tested by the referring veterinarian for FIV and FeLV using in-clinic ELISA tests (Snap Combo, IDEXX Lab, Westbrook, MA, USA) and were negative. Anti-FCoV serology was performed using an indirect immunofluorescence test on 10-multi-well slides produced at the University of Zurich by coating each well with 4.5×10^3 PD-5 cells, half of which were infected with swine transmissible gastroenteritis virus (TGEV), which serologically cross-reacts with FCoVs (Osterhaus et al 1977). Serology was performed as follows: serial two-fold dilutions of each serum sample (1:25 to 1:400) were prepared. Twenty microlitres of each dilution were applied to each well and incubated (30 min, 37 °C) in a humid chamber. Slides were then washed three times with PBS, dried, and 15 µl of FITC-conjugated rabbit-anti cat immunoglobulin (Nordic Immunological Laboratories, Tilburg, The Netherlands) were applied to each well. After incubation (30 min, 37 °C in a humid chamber), the slides were washed, dried, coverslipped with PBS and Kaiser's glycerin (1:3 ratio) and observed on a fluorescence microscope. The dilutions which showed a clear fluorescence signal in about one half of the cells were judged as positive. Samples that were still positive at a 1:400

dilution were further serially diluted on a two-fold basis until the appearance of a negative result.

Validation of a semi-quantitative approach to analyse RT-PCR products

In order to validate a semi-quantitative method to compare results obtained in different test sessions, we analysed the data recorded for IL-1 β , which in preliminary tests were shown to be expressed in all cats examined. As in all the PCR tests a 100 bp standard marker was included, a randomly chosen band of the mass ladder (the band corresponding to 500 bp in our case) was the ideal candidate to be used as an 'image calibrator'. Values were then expressed as percentages of mean intensity recorded for the 500 bp band, assuming that any possible variation in the amount of intensity recorded for the 500 bp likely depend on gel processing or image analysis procedures.

To verify the reliability of this approach a sample from the SPF cat H was included in each test session, all the samples collected in the second sampling (T2) were analysed twice, and randomly chosen cDNAs from different samplings were simultaneously run in additional batches of tests and re-analysed according to the above mentioned approach. Results from repeated tests of the same cat were used to calculate mean, standard deviation and coefficient of variation ($CV = (SD/mean) \times 100$) (Stockham and Scott 2002).

Analysis of cytokine profiles of FCoV-infected cats

All values regarding cytokine expression were evaluated by image analysis and the results were expressed with a traditional qualitative approach (positive or negative) and also with the semi-quantitative approach described above.

Semi-quantitative data recorded in the three groups of cats were compared against each other using the non-parametric Kruskal–Wallis test followed by the Bonferroni test. The same tests were used to compare (a) the results recorded in each sampling time of group 1 cats; (b) the results recorded in each sampling time of group 1 cats with those of other groups. The cytokine levels of cats with FCoV faecal shedding were compared with those of cats with negative faeces by using the Mann–Whitney *U* test. The possible presence of correlations between results of serology, haematology

and cytokine expression was investigated using the Spearman correlation test.

Results

Health status of the cats in the cattery, haematology, antibody titres and FCoV excretion

Cats from group 1 never showed clinical signs or haematological abnormalities attributable to FIP or to any other infectious disease during the sampling period. Nevertheless, 12 days after sampling 3 and 30 days after sampling 5, two episodes of FIP occurred in the cattery where they were living. Specifically, these episodes lead to the death of two kittens and one cat, respectively. Antibody titres and the amount of faecal excretion of FCoVs were also variable among the different cats and samplings (Table 3). No statistically significant correlations between antibody titres and haematological parameters were found.

Post-mortem examinations revealed that cat E was affected by a dry form of FIP, with severe neurological and renal lesions, while cats F and G had the typical abdominal effusive form. Haemograms revealed that cats with FIP were anaemic and had lymphocytosis (cat E) or lymphopenia (cats F and G). Antibody titres of cats with FIP were 1:800, 1:400 and 1:200, respectively. The healthy non-FCoV infected SPF cats showed no clinical signs or haematological change when sampled and they had no circulating anti-FCoV antibodies or FCoVs in faeces.

Validation of the semi-quantitative RT-PCR

The repeatability of the protocol and the possibility to compare the results obtained in different test sessions were investigated by normalising the results of image analysis of IL-1 β as a function of an 'image calibrator' for each batch of tests (see an example in Fig 1). The results of such a comparison are reported in Table 4. In the large majority of cases (10/12, 83%) the CV was lower than 15%. Specifically, four samples had a CV lower than 5%, four samples had a CV between 5% and 10%, and two additional cases had a CV between 10% and 15%. Only in cat C (sampling 3) and in cat H the CV was higher than 20%. The high CV of the latter sample, however, mostly results from just one sample (batch 2) that was significantly lower than the other four, which, on the contrary, provided very similar

Table 3. Results of serology, PCR on faeces, and qualitative cytokine expression in cats of group 1. Time intervals: 90 days from T1 to T2, 35 days from T2 to T3 and from T3 to T4 and 70 days from T4 to T5. Cases of FIP occurred 12 days after sampling 3 and 30 days after sampling 5

| Sampling number | Cat | Anti-FCoV | FCoV PCR | IL-1 β | IL-4 | IL-6 | IL-10 | IL-12 _{p40} | IFN γ | TNF α |
|-----------------|-----|-----------|----------|--------------|------|------|-------|----------------------|--------------|--------------|
| 1 | A | 1:100 | + | + | + | - | - | + | - | - |
| | B | 1:100 | + | + | - | - | - | + | - | - |
| | C | 1:100 | + | + | - | - | - | - | - | - |
| | D | 1:400 | + | + | + | - | - | - | - | - |
| 2 | A | 1:200 | + | + | - | - | - | - | - | - |
| | B | 1:1.600 | + | + | + | - | - | - | - | - |
| | C | 1:800 | + | + | + | - | + | - | + | - |
| | D | 1:50 | + | + | - | - | + | - | - | - |
| 3 | A | 1:400 | + | + | + | + | + | + | + | + |
| | B | 1:800 | - | + | + | - | - | + | - | - |
| | D | 1:400 | + | + | + | - | - | + | - | - |
| 4 | A | 1:400 | - | + | + | - | - | + | + | - |
| | C | 1:800 | + | + | - | - | - | + | - | - |
| | D | 1:50 | + | + | + | - | - | + | - | - |
| 5 | A | 1:3.200 | + | + | + | - | - | + | + | + |
| | B | 1:400 | - | + | + | - | - | - | + | - |
| | C | 1:3.200 | + | + | + | + | - | + | + | - |
| | D | 1:50 | + | + | - | - | - | + | + | + |

results (CV of the other four measurements was 7.6%).

Cytokine analysis and relationship between cytokine patterns and FCoV status

All the cats from group 1 expressed IL-1 β . IL-12_{p40} (Fig 2A) and IL-4 (Fig 2B) were detected

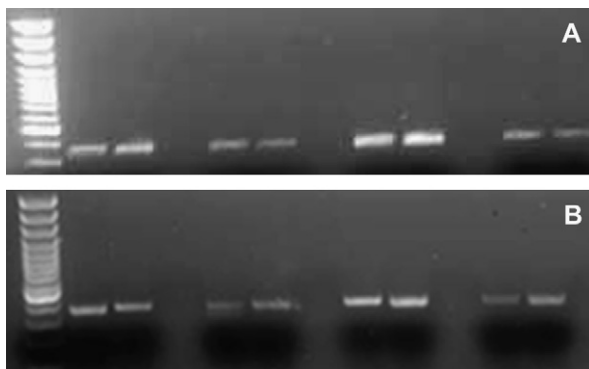


Fig 1. Repeatability of RT-PCR among different sessions of tests. RT-PCR products from sampling 2 of cats A (lanes 2 and 3), B (lanes 5 and 6), C (lanes 8 and 9), and D (lanes 11 and 12). Lane 1: 100 bp standard marker. The results here reported correspond to batches 2 (A) and 6 (B) of Table 4.

in the majority of samples. IFN- γ (Fig 2C), TNF- α and especially IL-6 and IL-10 were rarely detected (Table 3). Nevertheless, the number of cats expressing the different cytokines varied among the different samplings: specifically, cats expressing IL-4, IL-6, IL-12_{p40} and TNF- α were detected mainly on samplings 3 and 5 and all the cats expressed IFN- γ on sampling 5. On the contrary, cats expressing IL-10 were found only on samplings 2 and 3. Among the cats with FIP, cat E was positive for all the cytokines, except IL-6 and IL-10, cat F expressed only IL-1 β and IL-6, while cat G was positive only for IL-1 β . The SPF cats expressed only IL-1 β except for cat I which also expressed TNF- α .

The semi-quantitative analysis of RT-PCR results showed no significant differences among the levels of cytokines recorded in the different cats of group 1 or among the groups of cats (FCoV, FIP and SPF). On the contrary, statistical analysis revealed significantly higher levels of IL-1 β and IFN- γ in group 1, sampling 5, compared to group 1, sampling 1, and to both SPF and FIP cats ($P < 0.01$) (Tables 5 and 6). Cytokine levels were not different in cats that shed coronaviruses compared to those that had FCoV-negative faeces (data not shown). In group 1, the

Table 4. Scheme of the samples in which IL-1 β was repeatedly determined

| Cat | Sampling number | Batch 2 | Batch 3 | Batch 4 | Batch 5 | Batch 6 | Batch 7 | Batch 8 | Mean | SD | CV |
|----------|-----------------|---------|---------|---------|---------|---------|---------|---------|------|------|------|
| A (FCoV) | 2 | 68.8 | | | | 58.6 | | 65.5 | 64.3 | 5.2 | 8.1 |
| | 3 | | 85.8 | | | | 89.3 | | 87.5 | 2.5 | 2.9 |
| | 4 | | | 85.2 | | | | 94.6 | 89.9 | 6.7 | 7.4 |
| | 5 | | | | 91.4 | | | 92.6 | 92.0 | 0.8 | 0.9 |
| B (FCoV) | 2 | 47.6 | | | | 53.2 | | | 50.4 | 4.0 | 7.9 |
| | 3 | | 68.8 | | | | | 83.3 | 76.0 | 10.2 | 13.5 |
| | 4 | | | | 80.9 | | 72.1 | | 76.5 | 6.2 | 8.1 |
| C (FCoV) | 2 | 76.8 | | | | 79.9 | | | 78.4 | 2.2 | 2.9 |
| | 3 | | | 39.8 | | | 55.3 | | 47.6 | 11.0 | 23.1 |
| D (FCoV) | 2 | 46.1 | | | | 54.3 | | | 50.2 | 5.8 | 11.6 |
| | 3 | | 74.8 | | | | 77.4 | | 76.1 | 1.8 | 2.4 |
| H (SPF) | | 33.4 | 56.2 | | 65.0 | 55.2 | | 57.3 | 53.4 | 11.8 | 22.2 |

Values are expressed as percentage of the mean intensity of the internal calibrator (500 bp ladder) of each batch. The analysis of the repeatability of the computerised quantification of IL-1 β expression was performed by examining mean, standard deviation (SD) and coefficient of variation (CV). None of the samples included in batch 1 were repeatedly analysed so no columns referring to batch 1 are therefore included in this table.

level of IFN- γ expression was significantly correlated with the percentage of lymphocytes in the blood used for mRNA extraction ($P < 0.05$, $r = 0.75$) (Table 5).

Discussion

Compared with RT-PCR on isolated cell population, the analysis of whole blood has the advantage of a small number of ex-vivo manipulations and fewer effects on cytokine expression patterns and it thus provides more precise information about the 'peripheral immune status' of infected cats. It has been reported that standard RT-PCR has the same reproducibility for relative quantification of mRNA as for other methods like competitive RT-PCR (Hall et al 1998). Finally, the use of specific software to calculate band intensity is an easy and frequently used method for semi-quantitative analysis of digitalised gel images (Harley et al 1999, Yancy et al 2001, Duvigneau et al 2003). For these reasons we used a traditional RT-PCR technique to study cytokine profiles in feline whole blood and we quantified the bands' intensity by image analysis. This method allowed us to obtain repeatable and reliable results, thus indicating that it can be used to compare results from different test sessions. In order to correctly interpret the results, however, it is important to standardise some of the methodological factors: (1) time between blood collection and RNA extraction: it has

been reported that cold storage of whole blood poorly affects the amount of RNA extracted, even though it might activate some cell populations (Duvigneau et al 2003); nevertheless standardisation of processing times would affect all the samples in a similar manner; (2) amount of cells used for RNA extraction: this must be done by counting white blood cells in whole blood and using an appropriate amount of blood for RNA extraction; (3) amount of cDNA used in the PCR reaction: using the semi-quantitative method, the number of RNA copies in each band is unknown, but, based on the results of normalisation with GAPDH, the same amount of cDNA was loaded in each PCR reaction and, as an image calibrator was included in each gel, the relative amount of cytokines expressed in different samplings, in different batches of tests or in different groups of cats, can be compared to each other, as was the case in this study.

Using the above described approach, we examined whole blood cytokine profiles in cats with symptomatic and non-symptomatic FCoV infection and in healthy non-FCoV infected SPF cats. The use of SPF cats as a negative control might appear unusual as the immune system of these cats is exposed to stimuli (non-specific infectious agents, environmental stress) different from those of non-SPF cats. On the other hand, the few data available regarding feline cytokines have been recorded in SPF cats (Rottman et al 1995, Kipar et al 2001). Moreover, a control group composed by

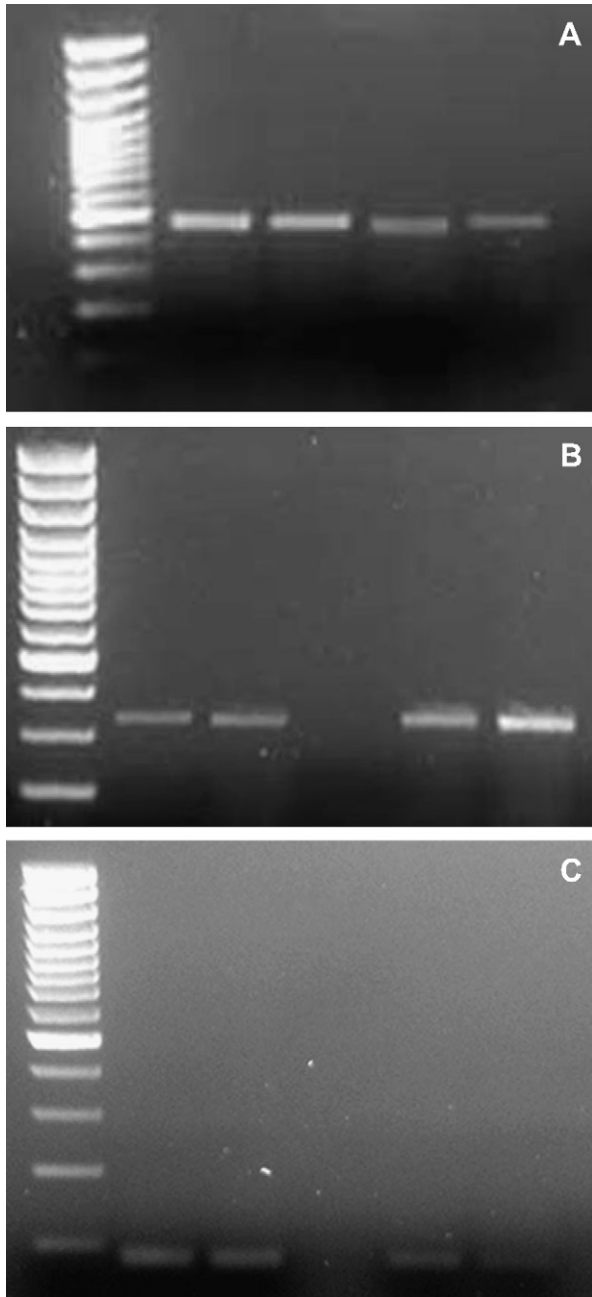


Fig 2. Examples of RT-PCR products for IL-12_{p40} (A) IL-4 (B) and IFN- γ (C) recorded in FCoV-exposed cats.

FCoV-negative non-SPF cats from FCoV-negative multi-cat households would have been a better control group, but it would not represent a frequent situation, in practice: only a low proportion of multi-cat households (less than 10% in our area) can be actually considered FCoV-negative, while the percentage of FCoV-infected animals in the large majority of catteries is close to 100% (Pedersen 1995). In group 1, we found both persistent and recurrent shedders with fluctuating

antibody titres, as already reported in FCoV-endemic catteries (Addie and Jarrett 2001), and post-mortem findings of cats from group 2 were consistent with FIP. The sampled population can thus be considered a good sample for studying naturally occurring FCoV–host interactions.

Only IL-1 β and, in one case, TNF- α , was detectable in the blood of the SPF cats, in partial agreement with previous reports, on which, however, TNF- α was consistently expressed, perhaps due to the isolation procedure inducing cell activation (Rottman et al 1995, Leutenegger et al 1999).

Cytokine expression in groups 1 and 2 was characterised by a high individual variability that was responsible for the lack of significant differences with SPF cats. This variability has been already reported (Gunn-Moore et al 1998, Foley et al 2003). It is unlikely that variability might depend on age, as reported by Kipar et al (2005), as cats of different age were present in all the groups, or on the different breed of cats belonging to the different groups, given that breed-associated variability in cytokine expression in cats has not been demonstrated.

The type and the amount of cytokines expressed by non-symptomatic FCoV-infected cats were not related to faecal shedding or to antibody titres, but prior to the appearance of FIP in the cattery both the percentage of clinically healthy FCoV-positive cats expressing most of the cytokines, and the median levels of IL-1 β and IFN- γ significantly increased. This is in agreement with the increases of certain lymphocyte subsets and acute phase proteins (Paltrinieri et al 2003, Giordano et al 2004), which might depend on the expression of the above cytokines, in cats that remain healthy when other cats of the cattery develop FIP. The positive correlation between IFN- γ expression and lymphocyte percentage might depend on the fact that non-symptomatic FCoV-infected cats have high numbers of CD8+ lymphocytes (Paltrinieri et al 2003), which can produce high levels of IFN- γ (Caruso et al 1998). All these findings suggest that an intense cytokine production by peripheral blood cells, and specifically an increased expression of IL-1 β and IFN- γ , might protect FCoV-infected cats from FIP (Kiss et al 2004). Ultimately, FIP occurs when low pathogenic FCoV strains mutate and acquire the ability to replicate within macrophages, but the probability of mutation depends on the rate of replication of enteric FCoVs (Vennema et al 1998). It is thus possible that viral replication is lower in cats that mount an efficient immunity.

Table 5. Lymphocyte percentage and cytokine expression, expressed as percentages of intensity of the internal calibrator included in the corresponding batch of tests. Values referred to each group were expressed as median, I and III quartiles, and 95° percentile ranges (between parentheses)

| Cat | Sampling number | Lymph % | IL-1 β | IL-4 | IL-6 | IL-10 | IL-12 _{p40} | IFN- γ | TNF- α |
|-------------------|-----------------|---------|----------------------------------|--------------------------------|-------------------------------|------------------------------|-------------------------------|-------------------------------|-------------------------------|
| A | 1 | 36 | 63.1 | 41.3 | 0.0 | 0.0 | 55.3 | 0.0 | 0.0 |
| | 2 | 38 | 64.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 3 | 38 | 87.5 | 51.1 | 50.1 | 19.7 | 72.4 | 8.3 | 61.1 |
| | 4 | 31 | 89.9 | 32.0 | 0.0 | 0.0 | 52.4 | 21.4 | 0.0 |
| | 5 | 35 | 92.0 | 39.7 | 0.0 | 0.0 | 72.5 | 26.4 | 62.3 |
| B | 1 | 42 | 40.1 | 0.0 | 0.0 | 0.0 | 61.5 | 0.0 | 0.0 |
| | 2 | 51 | 53.2 | 38.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 3 | 28 | 76.0 | 80.6 | 0.0 | 0.0 | 48.2 | 0.0 | 0.0 |
| | 5 | 52 | 76.5 | 42.3 | 0.0 | 0.0 | 0.0 | 23.2 | 0.0 |
| C | 1 | 25 | 56.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 2 | 50 | 80.0 | 36.0 | 0.0 | 53.2 | 0.0 | 31.6 | 0.0 |
| | 4 | 26 | 47.5 | 0.0 | 0.0 | 0.0 | 49.6 | 0.0 | 0.0 |
| | 5 | 39 | 93.8 | 50.4 | 41.7 | 0.0 | 82.0 | 27.3 | 0.0 |
| D | 1 | 38 | 31.7 | 48.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 2 | 35 | 54.3 | 0.0 | 0.0 | 58.2 | 0.0 | 0.0 | 0.0 |
| | 3 | 57 | 76.1 | 24.0 | 0.0 | 0.0 | 42.7 | 0.0 | 0.0 |
| | 4 | 35 | 71.6 | 40.8 | 0.0 | 0.0 | 52.8 | 0.0 | 0.0 |
| | 5 | 48 | 93.0 | 0.0 | 0.0 | 0.0 | 90.0 | 29.1 | 61.6 |
| Group 1 (FCoV) | | | 73.8 54.8–85.6 (35.3–93.5) | 37.1 0.0–42.1 (0.0–68.1) | 0.0 0.0–0.0 (0.0–46.5) | 0.0 0.0–0.0 (0.0–56.1) | 48.9 00–60.0 (0.0–86.6) | 0.0 0.0–22.8 (0.0–30.5) | 0.0 0.0–0.0 (0.0–62.0) |
| E | | | 87.1 | 20.4 | 0.0 | 0.0 | 78.7 | 7.4 | 65.7 |
| F | | | 20.8 | 0.0 | 27.2 | 0.0 | 0.0 | 0.0 | 0.0 |
| G | | | 34.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Group 2 (FIP) | | | 34.0 27.4–60.6 (21.5–84.4) | 0.0 0.0–10.2 (0.0–19.4) | 0.0 0.0–13.6 (0.0–25.8) | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–39.4 (0.0–74.8) | 0.0 0.0–3.7 (0.0–7.0) | 0.0 0.0–32.9 (0.0–62.4) |
| H | | | 53.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| I | | | 44.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 51.4 |
| L | | | 55.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Group 3 (SPF) | | | 53.4 48.8–54.7 (44.7–55.8) | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–25.7 (0.0–48.8) |

The cytokine expression pattern recorded in cats with FIP was not consistent, probably related to the different clinical presentations: a cytokine pattern consistent with activation of innate (IL-1 β and TNF- α) and cell-mediated (IL-12_{p40}) immunity was found in cat E, which had a dry form. By contrast, the cytokine profiles of cats F and G, which had the wet form, were quite silent: both these cats expressed only low levels of IL-1 β and cat F also expressed IL-6. A decreased cytokine expression was already reported, in blood, during experimental infections (Gunn-Moore et al 1998), but it is in contrast with that recorded in tissues (Dean et al 2003, Foley et al 2003) and in

ascites, on which high levels of IL-6 were recorded (Goitsuka et al 1990). Interestingly, all the cats with FIP had low or absent IFN- γ expression. Also IFN- γ has been found to be upregulated within lesions (Foley et al 2003, Berg et al 2005), but downregulated in blood of cats with FIP (Kiss et al 2004). Cytokine profiles in blood therefore seem to be different from those in tissues of cats with FIP. Moreover, the low levels of IFN- γ recorded in cats with FIP compared with the high levels recorded in FCoV-positive clinically healthy cats supports the hypothesis raised by Kiss et al (2004) about the key role of this molecule in predisposing to or in protecting from FIP.

Table 6. Semi-quantitative image analysis of RT-PCR products: median, I and III quartiles, and 95° percentile ranges (between parentheses) recorded in the five samplings of FCoV infected cats, in FIP cats and in SPF cats

| Cytokine | Sampling 1 | Sampling 2 | Sampling 3 | Sampling 4 | Sampling 5 | Group 2 (FIP) | Group 3 (SPF) |
|----------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|--|----------------------------------|----------------------------------|
| IL-1 β | 48.2 38.0–58.0 (32.3–62.6) | 59.3 54.0–68.2 (53.3–78.8) | 76.1 76.1–81.8 (76.0–86.9) | 71.6 59.6–80.8 (48.7–89.0) | 92.5* \S 88.1–93.2 (77.7–93.7) | 34.0 27.4–60.6 (21.5–84.4) | 53.4 48.8–54.7 (44.7–55.8) |
| IL-4 | 20.7 0.0–43.1 (0.0–47.9) | 18.0 0.0–36.6 (0.0–38.0) | 51.9 37.6–65.9 (51.1–79.1) | 32.0 16.0–36.4 (1.6–40.4) | 41.0 29.8–44.3 (3.0–49.8) | 0.0 0.0–10.2 (0.0–19.4) | 0.0 0.0–0.0 (0.0–0.0) |
| IL-6 | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–0.0 (0.0–0.0) | 16.7 0.0–25.1 (0.0–47.6) | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–10.4 (0.0–38.6) | 0.0 0.0–13.6 (0.0–25.8) | 0.0 0.0–0.0 (0.0–0.0) |
| IL-10 | 0.0 0.0–0.0 (0.0–0.0) | 26.6 0.0–54.5 (0.0–57.8) | 6.6 0.0–9.9 (0.0–18.7) | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–0.0 (0.0–0.0) |
| IL-12 _{p40} | 27.7 0.0–56.9 (0.0–61.0) | 0.0 0.0–0.0 (0.0–0.0) | 54.4 45.5–60.3 (48.2–71.2) | 52.4 51.0–52.6 (49.7–52.8) | 77.3 54.4–84.0 (5.4–89.4) | 0.0 0.0–39.4 (0.0–74.8) | 0.0 0.0–0.0 (0.0–0.0) |
| IFN- γ | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–7.9 (0.0–29.2) | 2.8 0.0–4.2 (0.0–7.9) | 0.0 0.0–10.7 (0.0–20.3) | 26.9* \S 25.6–27.8 (23.4–29.0) | 0.0 0.0–3.7 (0.0–7.0) | 0.0 0.0–0.0 (0.0–0.0) |
| TNF- α | 0.0 0.0–0.0 (0.0–0.0) | 0.0 0.0–0.0 (0.0–0.0) | 20.4 0.0–30.6 (0.0–58.0) | 0.0 0.0–0.0 (0.0–0.0) | 30.8 0.0–61.8 (0.0–62.2) | 0.0 0.0–32.9 (0.0–62.4) | 0.0 0.0–25.7 (0.0–48.8) |

* $P < 0.05$ vs sampling 1. $\S P < 0.01$ vs FIP, SPF.

In conclusion, RT-PCR analysis of cytokine patterns in whole blood can be used to evaluate the immune status of cats. The semi-quantitative approach based on the analysis of digitalised gel images provides repeatable results. As the analysis of whole blood is considered to be highly representative of the in vivo situation, our results further support the hypothesis that upregulation of cytokines by blood cells might protect FCoV-infected cats from FIP, while down-regulation of cytokines (and especially of IFN- γ) is associated with FIP. These results must be verified by larger and well controlled studies to investigate both the role of cytokines in FCoV–host interactions and the possibility to use cytokine profiles to identify cats susceptible or resistant to FIP.

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References

- Addie DD, Jarret JO (2001) Use of reverse-transcriptase polymerase chain reaction for monitoring feline coronavirus shedding by healthy cats. *Veterinary Record* **148**, 649–653.
- Allen JN, Herzyk DJ, Allen ED, Wewers MD (1992) Human whole blood interleukin- β production: kinetics, cell source and comparison with TNF- α . *Journal of Laboratory and Clinical Medicine* **119**, 538–546.
- Balkwill FR, Burke F (1989) Cytokine network. *Immunology Today* **10**, 299–304.
- Berg A-L, Ekman K, Belák S, Berg M (2005) Cellular composition and interferon- γ expression of the local inflammatory response in feline infectious peritonitis (FIP). *Veterinary Microbiology* **111**, 15–23.
- Caruso A, Lincenziati S, Morelli D, Fiorentini S, Ricotta D, Malacarna F, Sfondrini L, Balsari A (1998) Segregation of type 1 cytokine production in human peripheral blood lymphocytes: phenotypic differences between IFN- γ and IL-2 producing cells in the CD8+T cell subset. *European Journal of Immunology* **28**, 3630–3638.
- Dean GA, Olivry T, Stanton C, Pedersen NC (2003) In vivo cytokine response to experimental feline infectious peritonitis virus infection. *Veterinary Microbiology* **97**, 1–12.
- DeLaurier A, Allen S, deFlandre C, Horton MA, Price JS (2002) Cytokine expression in feline osteoclastic resorptive lesions. *Journal of Comparative Pathology* **127**, 169–177.

- Duvigneau JC, Hartel RT, Teinfalt M, Gemeneir M (2003) Delay in processing porcine whole blood affects cytokine expression. *Journal of Immunological Methods* **272**, 11–21.
- Foley JE, Rand C, Leutenegger C (2003) Inflammation and changes in cytokines levels in neurological feline infectious peritonitis. *Journal of Feline Medicine and Surgery* **5**, 1–10.
- Giordano A, Spagnolo V, Colombo A, Paltrinieri S (2004) Changes in some acute phase protein and immunoglobulin concentrations in cats affected by feline infectious peritonitis or exposed to feline coronavirus infection. *Veterinary Journal* **167**, 38–44.
- Goitsuka R, Ohashi T, Ono K, Yasukawa K, Koishibara Y, Fukui H, Ohsugi Y, Hasegawa A (1990) IL-6 activity in feline infectious peritonitis. *Journal of Immunology* **144**, 2599–2603.
- Gunn-Moore DA, Caney SMA, Gruffydd-Jones TJ, Helps CR, Harbour DA (1998) Antibody and cytokine response in kittens during the development of feline infectious peritonitis (FIP). *Veterinary Immunology and Immunopathology* **65**, 221–242.
- Hall LL, Bicknell GR, Primrose L, Pringle JH, Shaw JA, Furness PN (1998) Reproducibility in the quantification of mRNA levels by RT-PCR and RT competitive-PCR–ELISA. *BioTechniques* **24**, 652–658.
- Ham JM, Kunkel SL, Dibb CR, Standiford TJ, Rolfe MW, Strieter RM (1991) Chemotactic cytokine (IL8 and MCP-1) gene expression by human whole blood. *Immunological Investigations* **20**, 387–394.
- Harley R, Helps CR, Harbour DA, Gruffydd-Jones TJ, Day MJ (1999) Cytokine mRNA expression in lesions in cats with chronic gingivostomatitis. *Clinical and Diagnostic Laboratory Immunology* **6**, 471–478.
- Härtel C, Bein G, Müller-Steinhardt M, Klüter H (2001) Ex vivo induction of cytokine mRNA expression in human blood samples. *Journal of Immunological Methods* **249**, 63–71.
- Herrewegh AAPM, Egberink HF, Horzinek MC, Rottier PJM, De Groot RJ (1995) Polymerase chain reaction (PCR) for diagnosis of naturally occurring feline coronavirus infections. *Feline Practice* **23**, 56–60.
- Kipar A, Baptiste K, Meli ML, Barth A, Knietzsch M, Reinacher M, Lutz H (2005) Age-related dynamics of constitutive cytokine transcription levels of feline monocytes. *Experimental Gerontology* **40**, 243–248.
- Kipar A, Leutenegger CM, Hetzel U, Akens MK, Mislin CN, Reinacher M, Lutz H (2001) Cytokine mRNA levels in isolated feline monocytes. *Veterinary Immunology and Immunopathology* **78**, 305–315.
- Kiss I, Poland AM, Pedersen NC (2004) Disease outcome and cytokine responses in cats immunized with an avirulent feline infectious peritonitis virus (FIPV)-UCD1 and challenge-exposed with virulent FIPV-UCD8. *Journal of Feline Medicine and Surgery* **6**, 89–97.
- Leutenegger CM, Mislin CN, Sigrist B, Ehrenguber U, Hofmann-Lehmann R, Lutz H (1999) Quantitative real-time PCR for the measurement of feline cytokine mRNA. *Veterinary Immunology and Immunopathology* **71**, 291–305.
- Mosmann TR, Sad S (1996) The expanding universe of T-cell subset: Th1, Th2 and more. *Immunology Today* **17**, 138–146.
- Osterhaus AD, Horzinek MC, Reynolds DJ (1977) Seroepidemiology of feline infectious peritonitis virus infections using transmissible gastroenteritis virus as antigen. *Zentralblatt für Veterinärmedizin [B]* **24**, 835–841.
- Paltrinieri S, Ponti W, Comazzi S, Giordano A, Poli G (2003) Shifts in circulating lymphocyte subsets in cats with feline infectious peritonitis (FIP): pathogenic role and diagnostic relevance. *Veterinary Immunology and Immunopathology* **96**, 141–148.
- Pedersen NC (1995) An overview of feline enteric coronavirus and infectious peritonitis virus infections. *Feline Practice* **23**, 7–20.
- Rottman JB, Freeman EB, Tonkonogy S, Tompkins MB (1995) A reverse transcription-polymerase chain reaction technique to detect feline cytokine genes. *Veterinary Immunology and Immunopathology* **45**, 1–18.
- Stockham SL, Scott MA (2002) Fundamentals of Veterinary Clinical Pathology. Iowa: Iowa state press.
- Stordeur P, Zhou L, Goldman M (2002) Analysis of spontaneous mRNA cytokine production in peripheral blood. *Journal of Immunological Methods* **261**, 195–197.
- Vennema H, Poland A, Foley J, Pedersen NC (1998) Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. *Virology* **243**, 150–157.
- Yancy H, Ayers SL, Farrell E, Day A, Myers MJ (2001) Differential cytokine mRNA expression in swine whole blood and peripheral blood mononuclear cell cultures. *Veterinary Immunology and Immunopathology* **79**, 41–52.