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Evaluation of in vitro diagnostic (point-of-care) system for quantification of the acute phase protein haptoglobin in cats

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To my parents Britt-Marie and Rolf Mattsson,
and my sisters Terese Österholm and Linn Mattsson.
Your Jennifer

"Logic will get you from A to B.
Imagination will take you everywhere."
Albert Einstein.

Glossary/Abbreviations:

In vitro: cells growing in a context outside their organism

Point of care test: Test near the patient or inhouse test

Haptoglobin: plasma protein, an acute phase protein

CRP: C-reactive protein

SIRS: systemic inflammatory response syndrome

MODS: multiple organ dysfunction syndrome

IL-1: inflammatory cytokine

IL-6: pro-inflammatory cytokine / anti-inflammatory myokine)

TNF-a: tumor necrosis factor-alpha

SAA: serum amyloid A, an acute phase protein

AGP: alpha 1-acid glycoprotein, an acute phase protein

T lymphocytes: type of white blood cells

B lymphocytes: type of white blood cells

MV: mean value

Abstract

This thesis consists of an evaluation of a new *in vitro* diagnostic point-of-care system for the quantification of the acute phase protein haptoglobin (Feline Haptoglobin, LifeAssays®). Acute phase proteins are biomarkers of systemic inflammation and can thus be used for diagnosis and monitoring of inflammation [1]. The evaluation was conducted on 79 existing serum samples from both healthy and sick cats analyzed with LifeAssays point of care system. The samples came from cats of different age, breed and gender, and were divided into three categories: healthy; diseased with systemic inflammation, and sick with other diseases. The findings show close agreement between the haptoglobin concentration as measured by LifeAssays system and the patients' clinical conditions. The clinical sensitivity was determined to be 91%, and clinical specificity 100%.
Keywords: cat, diagnostics, acute phase protein, haptoglobin

Background

SIRS

Systemic inflammatory response syndrome (SIRS) is an indication of increasing stimuli able to cause the release of inflammatory mediators. Any infection or injury can cause SIRS, e.g. liver diseases, burns, stroke or septicemia. Early detection is extremely important when it comes to complications that can occur due to SIRS. The risk of being suffering multiple organ dysfunction syndrome (MODS) is high among these patients and mortality thereby increases dramatically [2]. Even a patient with minor SIRS symptoms risk of MODS if that patient has a relapse beause the immune system reacts to minor infections. This is called the "two hit" theory. Development from SIRS to MODS is probably due to an imbalance between the two pro-inflammatory cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNFa) [3]. The threshold values used as criteria for humans are also applied to dogs as well as cats. The criteria for SIRS have been reported previously by Purvis & Kirby (1994) [1], Hardie (1995) [4] and Hauptman, Walshaw & Oliver (1997) [5] and are shown below *(Table 1).*

Table 1. The table shows the criteria that form the basis for systemic inflammatory response syndrome (SIRS) in cats. Three different studies show similar results.

Criteria	Purvis & Kirby (1994)	Hardie (1995)	Hauptman, Walshaw & Oliver (1997)
Heart rate (beats/min)	>20	>20	>20
Respiratory rate (breaths/min)	>160	>120	>120
Temperature (C°)	<37.8, >39.7	<38.0, >40.0	<38.1, >39.2
White blood cells; Band neutrophils (x 10^3/µL)	<4, >12; >10%	<5, >18; >5%	<6, >16; >3%

Acute phase proteins

When subjected to injury or infection, the body reacts with a series of protection mechanisms: it creates a systemic inflammation leading to fever, increases the number of white blood cells (WBC), and the liver begins to release acute phase proteins into the blood. The concentration of these acute phase proteins in the blood thus rises and the increase can be used to detect infection at an early stage. An anti-inflammatory treatment can similarly be monitored by following changes in the concentration of the acute phase proteins. Early identification of an inflammation and follow-up of the efficacy of a potential treatment can limit unnecessary suffering in the patient and achieve a more economical process [1].

Acute phase proteins send signals to the acquired immune system to act rapidly against tissue damage and prevent the spread of bacteria in the host. Acute phase proteins can be divided into two groups: positive and negative. The negative proteins are reduced in the blood when there is inflammation, while the positive proteins increase in inflammation. The most important acute phase proteins in cats are serum amyloid A (SAA), alpha 1-acid glycoprotein (AGP) and haptoglobin [1].

Depending on the extent of the acute phase proteins' response to stimuli, they may be divided into three subgroups: major, moderate and minor. In cats, SAA and AGP are major acute phase proteins

and can increase 10-100-fold during an infection. Haptoglobin is a moderate acute phase protein and levels may be 2-10 times greater during infection [1].

The function of AGP is not entirely clear, but it is a protein that likely inhibits neutrophils, phagocytes and thrombocytes from doing their job. In addition, AGP plays a part in the maturation of T- and B lymphocytes. Concentrations increase slowly in cats but, once high, levels remain elevated for a longer time. AGP increases during surgical treatment, and in the presence of tumors, inflammation and infection [1].

SAA is a protein that, through chemotaxis, attracts neutrophils, T-cells and monocytes to a tissue injury site. SAA plays a part in opsonization and activates thrombocytes. The SAA protein also protects against endotoxins in the intestine. SAA is the protein that increases fastest in cat blood.

Haptoglobin is a protein that binds free hemoglobin in plasma. Free hemoglobin in plasma is toxic because of its ability to generate reactive oxygen species, but when haptoglobin binds hemoglobin this toxic reaction stops. The concentration of haptoglobin increases in all inflammatory diseases in cats *Table 2).* Hemolytic conditions, on the other hand, will be reflected in a decrease of haptoglobin levels. [1].

LifeAssays® Feline Haptoglobin System

The LifeAssays® Feline Haptoglobin *in vitro* diagnostic system consists of a small instrument, disposable reagent vials, and a chip with specific reagent data and algorithms. The system uses a linear measuring range for Feline Haptoglobin of 40–600 mg/dL and every analysis takes 11 minutes to execute. The reagent is based on a heterogeneous two-site immunoassay, based on the following principle: the reagent in the vial contains paramagnetic nanoparticles and larger silica particles. Both the magnetic nanoparticles and the silica particles are coated with antibodies that can bind the specific analyt to be quantified. When the analyte sample has been added, the magnetic material in the pellet in the reagent vial is enriched. The greater the concentration of the analyte in the sample, the more magnetic material in the pellet. The vial is then placed in the instrument to measure the paramagnetism in the reagent pellet, the measured value is an absolute concentration value of the analyte).

How a LifeAssays® Feline Haptoglobin system analysis is performed in practice is described in more detail in the instructions for use *(Appendix 1: LifeAssays® Feline Haptoglobin Test Kit).*

The Evaluation

The chosen aim for the evaluation was the determination of the clinical sensitivity (ability to find truly sick patients) and the clinical specificity (ability to find truly healthy patients). This is done based on the presence or absence of systemic inflammation and associated concentrations of haptoglobin.

Materials and methods

Serum capillary (Sarstedt, Nümbrecht, Germany).
Cat serum samples from Evidensia, Malmö Veterinary Hospital. Refrigerated samples were analyzed on the same day or the day after the serum sample was taken. Frozen samples were analyzed within one week of the day the serum sample was taken. All samples were drawn for purposes other than this evaluation.
LifeAssays® Feline Haptoglobin instrument 13-RD0005-01.
LifeAssays® Feline Haptoglobin reagent RD-199 & I-1364 with accompanying user manual.

The samples were analyzed as single samples on LifeAssays® Feline Haptoglobin system by the same person throughout the entire evaluation.

Case histories for the samples were printed out and the haptoglobin results were noted. The cats' white blood cell counts, temperature, case history and albumin levels were used to identify whether a patient had systemic inflammation or not, or was sick due to other causes *(Table 3,4,5)*.

Table 2. The table lists examples of diseases that can cause other elevated values of acute phase proteins in cats.

	Disease	Acute phase protein	Reference
Inflammation	Pancreatitis Kidney collapse Abscess Liver diseases, injuries Surgery	SAA SAA AGP, Hp SAA AGP, SAA, Hp	Tamamoto 2009 [6]. Sasaki 2003 [7]. Ottenjann 1996 [8]. Sasaki 2003 [7]. Kajikawa 1999 [9].
Bacteria	*Chlamydophila psittaci*	AGP	Terwee 1998 [10].
Virus	Feline infectious peritonitis	AGP, SAA, Hp	Duthie 1997 [11]. Giordano 2004 [12]. Paltrinieri 2007 [13].
Neoplasm	Lymphoma	SAA, AGP	Selting 2000 [14].
Endocrine system	Hyperthyroidism Diabetes mellitus	SAA SAA	Sasaki 2003 [7]. Tamamoto 2009 [6].
Autoimmune system	Autoimmune hemolytic anemia	SAA	Paltrinieri 2007 [13].

The haptoglobin cut-off chosen to calculate the clinical sensitivity and specificity was 250 mg/dL [14].

Results

A total of 79 cat samples were analyzed. Among these, 8 cats were healthy *(Table 3)* and 34 cats had SIRS *(Table 4)*. 30 additional cats were also diseased, but not with SIRS *(Table 5)* and 7 test samples were hemolyzed *(Table 6)*; the values from these two patient groups were accordingly not included in the data referring to clinical sensibility and clinical specificity. Samples that show traces of hemolysis, an indication of abnormality, show an erroneous haptoglobin concentration (see instructions for use in Appendix 1: LifeAssays®Feline Haptoglobin Test Kit).

1. The findings showed that 31 of the 34 cats with SIRS had haptoglobin concentrations over 250 mg/dL. Thus, 91.1% of the SIRS-diseased patients were identified. This patient group had a mean haptoglobin value of 452.2 mg/dL.

2. The findings showed that all of the 8 healthy cats had haptoglobin values below 250 mg/dL. Thus, 100% of the healthy patients were identified. This patient group had a mean haptoglobin value of 92 mg/dL.

3. The difference in haptoglobin concentrations between the SIRS group and the healthy cats was statistically significant. The null hypothesis, i.e. that there is no difference between the groups, was therefore rejected (p-value = $9.5 * 10^{-12}$).

Table 3. The table shows the analyzed values of the healthy cats. The clinical specificity was 100%.

Patient	Haptoglobin mg/dl	Temp C°	WBC $*10^9$/L	Albumin g/L
1	40*	-	-	27
2	58	-	4.9	31
3	40*	-	9.8	27
4	166	-	-	-
5	193	-	7.5	29
6	40*	-	11.3	39
7	106	-	-	34
8	93	37.5	8.8	28
MV	92	37.5	8.5	30.7

* The value of 40 mg/dL was applied for values <40 mg/dL in the calculations.

Table 4. The table shows the analyzed values of the SIRS diseased cats. The clinical specificity was 91%.

Patient	Haptoglobin mg/dL	Temp C°	WBC *10^9/L	Albumin g/L
1	600*	38.3	21.6	-
2	600*	39.2	7.2	32
3	298	-	11.7	24
4	600*	-	25.1	29
5	600*	-	8.9	36
6	600*	-	18.2	30
7	394	39.1	28.5	26
8	600*	38.9	15.8	24
9	600*	36.7	17.71	33
10	254	-	14.3	33
11	348	38.2	11.8	29
12	600*	36.7	26.2	-
13	358	35.8	16	20
14	319	-	3.2	21
15	456	40.4	8.1	45
16	600*	40.6	7.9	26
17	600*	37.2	7.9	34
18	390	39.7	-	28
19	226	37.9	4.2	-
20	301	-	6.5	32
21	600*	38.1	11.8	29
22	298	-	11.7	24
23	181	-	6.1	27
24	243	-	10.9	34
25	338	38.7	7.5	33
26	345	37.8	7.9	26
27	600*	36.1	33.8	30
28	600*	37.2	22.6	26.0
29	499	38.5	14.2	29.0
30	280	37.7	37.5	26.0
31	257	38.3	11.6	
32	600*	38.7	16.2	
33	600*	39.7		36.0
34	600*	38.2		31
MV	452.5	38.2	14.6	29.4

* The value of 600 mg/dL was applied for values >600 mg/dL in the calculations.

Table 5. The table shows the analyzed values of diseased cats, but that did not have SIRS.

Patient	Haptoglobin mg/dL	Temp C°	WBC *10^9/L	Albumin g/L
1	213	-	15.6	27
2	220	37.2	7.2	26
3	175	-	11.7	29
4	139	37.4	3.4	35
5	40*	-	-	-
6	77	-	11.9	28
7	193	-	5.2	29
8	86	-	10.2	35
9	217	-	8.3	30
10	89	-	-	-
11	178	37.8	9.6	26
12	189	-	-	-
13	40*	-	13.2	28
14	197	37.5	6.9	28
15	176	-	17.3	34
16	80	-	6.1	29
17	216	-	11.7	-
18	166	37.9	20.5	29
19	172	38.6	8.1	-
20	100	-	4.7	34
21	146	37.5	4.7	30
22	40*	38.6	8.1	-
23	600*	37.6	10.7	29
24	593	-	4.2	30
25	288	-	10.6	27
26	178	-	4.8	27
27	40*	-	-	27
28	153	-	9.4	25
29	216	-	7.4	-
30	189	-	4.3	35
MV	180.2	37.8	9.1	29.4

* In the calculations, the values of 40 mg/dL and 600 mg/dL were applied for values <40 mg/dL and >600 mg/dL, respectively.

Table 6. The table shows the samples analyzed that were hemolyzed. These were not included in the study results as the test results can be affected by hemolysis.

Patient	Haptoglobin mg/dL	Temp C°	WBC *10^9/L	Albumin g/L
1	<40	-	-	-
2	239	-	10.4	27
3	277	39	6.1	29
4	328	-	-	-
5	285	38.5	9.7	30
6	502	37.6	-	-
7	319	38.4	-	-

Discussion

The results are based on analysis of patient samples from cats of varied breeds, gender and states of health. The clinical specificity is 100%, which can, of course, not be better. The clinical sensitivity proved to be 91%, which is highly relevant and reasonable. Both clinical specificity and sensitivity are naturally affected by when the sample is taken in relation to the phase of systemic inflammation the patient is in.

Within the context of this thesis, a total of 79 samples were included, of which 34 were from patients with systemic inflammation. One way to further increase the statistical reliability could be to examine a greater number of samples.

In the calculations, a haptoglobin cut-off of 250 mg/dL was used. If this had only been decreased to 225 mg/dL, the clinical sensitivity would have increased to 97%. The clinical specificity, on the other hand, would have remained at 100%. The cut-off value is exactly the same as that used by Kann in her study entitled *Acute phase proteins in healthy and sick cats* [15]. The close agreement is probably due to the Feline Haptoglobin analysis with Tridelta's Elisa Peroxidase method used, since the LifeAssays® system is also calibrated using the reference values from Tridelta's method. Moreover, hemolyzed samples were also excluded in this study, which is considered relevant [15].

Regarding the clinical picture, the article entitled *Acute phase protein as biomarkers in animal health and welfare* [16] deals more specifically with diseases with increased haptoglobin in connection with SIRS. Here we see that the diseases in this project are in close agreement with the findings presented in the article. Mainly dominating were liver damage, kidney damage and the feline infectious peritonitis virus in the diseased cats. A small number of the SIRS cats suffered from the bacterium

Chlamydophila psittaci. The reason why there were so few of them is probably due to the fact that the bacterium is not as common as the other diseases and causes.

Unreliable measurements due to analytical factors such as precision and accuracy contribute to a spread in the results with all methods of analysis. In addition, there may also be errors due to pre-analytical factors. One way to reduce the overall measurement unreliability is to analyze a sample several times and then use the mean value. To simulate a typical user situation in this evaluation, however, all samples have been analyzed as single samples. This makes it important to take all clinical symptoms and other analysis results into consideration, not least for patient samples showing results near the cut-off for Feline Haptoglobin, before establishing a diagnosis.

Based on the results, the method of analysis evaluated can be expected to be of value for monitoring the treatment of patients with systemic inflammation. Nevertheless, sequential measurements of Feline Haptoglobin on selected patients were not included in this evaluation.

Conclusions

The results confirm that the method of analysis for Feline Haptoglobin evaluated can be used to identify cats with and without systemic inflammation. The method could thus prove to be a useful tool for correct diagnosis.

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