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Evaluation of vBA bile acids on Immuno AU10, Fuji film-preliminary results

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Bile acids are important measurands for detection of liver dysfunction in dogs and cats. For assessment of bile acid serum concentrations, several generations of assays are on the market. The enzymatic method (so called 3rd generation assay) requiring manual reconstitution of lyophilized material is therefore mainly used in small laboratories. The reaction is based on oxidation of bile acids catalyzed by the enzyme 3-α-hydroxysteroid dehydrogenase. Oxidized bile acids induce formation of NADH from NAD+ which further reacts with the dye nitrotetrazolium blue to a formazan dye, which can be detected at a wavelength of 540 nm.

In contrast, the enzyme cycling method (5th generation method) is liquid and does not require manual steps. It is thus widely used in large clinical laboratories. In the 5th generation bile acid assay, signal amplification is achieved by repeated oxidation and reduction of bile acids by the enzyme 3-α-hydroxysteroid dehydrogenase resulting in an accumulation of reduced co-enzyme thio-NADH that is measured at 405 nm. Due to the amplification steps, the test requires less sample volume than 3rd generation tests and is considered of superior analytical performance when compared to conventional bile acid assays.

Overall, reports about evaluation of point of care analysers (POCA) are scarce in veterinary medicine and only semiquantitative assays were evaluated using an antibody detecting canine bile acids which is linked to an enzyme catalyzing a color reaction (Seibert et al. 2014).

Recently, a novel quantitative point of care bile acid test (DRI-CHEM, Immuno AU10V v-BA test, Fujifilm Cooperation, Tokyo, Japan) run on the point of care analyzer Immuno AU10V (Fujifilm Cooperation, Tokyo, Japan) has been developed for rapid determination of canine and feline bile acids, which has not been evaluated before.

The assay utilizes a small cartridge in which the measurand is assessed with a competitive immunoassay using an antigen-antibody reaction which is augmented by so called Surface Plasmon enhanced Fluorescence (SPF), which allows the detection of small quantities of antigen (Li et al. 2017). Briefly, the bile acids in the sample bind to a fluorescent particle -labeled anti-bile acid mouse monoclonal antibody which is then attaching to bovine serum albumin attached to a thin gold film. Free particles, not bound to antibodies, do not show fluorescence activity, so that a washing step to keep background noise low, is not required. Then, laser irradiation is performed in an optimal angle to generate a resonance reaction by induction of waves on the film to resonate

with compressional waves of free electrons (so called surface plasmons) to generate a Surface Plasmon enhanced Resonance SPR to enhance fluorescence intensity which is then measured and is inversely proportional to the bile acid concentration in the specimen.

The use of both SPR and SPF has the aim to acquire signal amplification and noise reduction and is thus a highly sensitive method of detection of the analyte in comparison to conventional immuno-assays (Seibert et al. 2014).

The aims of the current study are:

To evaluate

1) Performance characteristics of the point-of-care analyser (POCA) Immuno AU10V for detection of canine and feline total bile acid serum concentration and

2) to characterize the clinical behavior of bile acids in approximately 10 cases (10 cats and dogs each) with various liver diseases including inflammatory and neoplastic liver diseases, liver cirrhosis and portosystemic shunt (PSS).

Our hypothesis was that the measurement of bile acids using the Fuji Immuno AU10V is technically easy and compares well especially with the 5^{th} generation bile acid assay run on the bench top analyser used as reference method.

Material and methods:

The study was performed between September 2017 and June 2018.

Part I: Performance characteristics evaluation

Evaluation of performance of the Immuno AU10V included assessment of linearity, interferences, lower limit of quantification, intra- and inter-assay precision and a method comparison study. For the method comparison study, samples of healthy and diseased dogs and cats (n=60 of each species planned) submitted to the central laboratory, faculty of veterinary medicine, Justus-Liebig-university Giessen, Germany were included, whereby 20 samples with normal to mildly increased bile acids (0-30 µmol/l), 20 samples with moderately increased bile acids (31-80 µmol/l) and 20 samples with markedly increased bile acids (> 80 µmol/l) were enrolled. Classification of bile acids in 3 concentration levels was performed as published previously (Tisdall et al. 1995).

The study was performed with samples of dogs and cats submitted for routine diagnostic work up to the central laboratory. Ethical approval to take an additional serum tube was given by the Regierungspräsidium Giessen, Germany **V54-19c2015h02GI18/17kTV10/2017.**

Pretreatment of the dogs and cats with ursodesoxycholic acid resulted in exclusion from the study.

I. Method validation

Linearity was assessed with pooled canine and feline serum.

To avoid a matrix effect due to extensive dilution of the serum sample with H_2O, stock solution was diluted serially so that always the same volume of stock solution (i.e, 10 µl) was added to the sample (i.e, 890 µl) to obtain different dilution steps.

For preparation of the stock solution, 25mg sodium hydrate cholate (Sigma, ca 1254-100g, soluble at 100 mg/ml; 1 mol=430.55 g) was dissolved in 2ml H_20 such that a stock solution containing 0.0290325 mol/L (=0.029 µmol/µl = 0.290 µmol/10µl) was obtained. Adding 10 µl of stock solution to 890 µl pool serum resulted in 900 µl serum containing 0,290325 µmol bile acids/900 µl = 322,58 µmol/l.

By serial dilution of the stock solution, specimen with 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125, and 0.00625 of the original bile acid concentration of the spiked sample were obtained. All samples, spiked with serially diluted stock solutions were analyzed in triplicates with the Immuno AU10V. For the sake of comparison, the remainder sample spiked with serially diluted stock solution was analyzed once with the 5^{th} generation bile acid test run on the Pentra bench top analyzer (Pentra-LT).

Precision and lower limit of quantification

Precision was assessed at three levels of bile acid concentration, i.e., low, moderate and high, whereby two samples at each concentration level were included.

Intra- and inter-assay Variation were calculated from replicate measurements performed with pooled serum samples.

For assessment of intra-assay CV, ten replicate measurements were performed. Inter-assay CV was calculated from measurements performed on seven consecutive days.

For assessment of LloQ, two canine serum samples with bile acid concentrations close to zero (6.2 µmol/L and 3.8 µmol/L) were measured 20 times in a single run.

Interferences

Potential interferences were assessed in aliquots à 960 µl of canine and feline pooled serum spiked with 40 µl of the interfering substance, i.e., hemoglobin, triglycerides, and bilirubin. The same volume of pooled serum sample was spiked with 40 µl of the respective diluent used to prepare the stock solution and dilution of the interfering substance, i.e., either pure double-

distilled water (in case of Intralipid), 0.09% NaCl (in case of hemoglobin), and 100 mM NaOH (in case of bilirubin), respectively.

Samples were analyzed in triplicates in random order.

For assessment of the possible impact of lipemia, hemolysis, and hyperbilirubinemia, 960 µl of a pooled canine and feline serum sample with a mean concentration of approximately 5 µmol/L bile acids was spiked with 8 g/L 20% soy bean emulsion (Intralipid 20%, Fresenius Kabi Canada, Ontario, Canada), 4 g/L hemoglobin (hemoglobin from bovine blood, lyophilized powder, Sigma-Aldrich Co. LLC., St. Louis, Missouri, USA), and 800 mg/L bilirubin (Bilirubin - ≥98%, powder, Sigma-Aldrich Co. LLC., St. Louis, Missouri, USA).

To evaluate the possible impact of lipemia on results, 40 µl of a stock solution containing 200 mg/ml were added to 960 µl of serum so that a concentration of soy bean oil of 8 g/L was obtained. If necessary, stock solution was further diluted such that a final concentration of 4 g/L (dog) and 2 g/L (cat) was obtained.

A stock solution containing 100 g/L hemoglobin was obtained by diluting 30 mg lyophilized bovine hemoglobin in 0.3 ml 0.09% NaCl. Subsequently, 40 µl of the solution were added to 960 µl non-spiked serum sample resulting in a hemoglobin concentration of 4 g/L.

For evaluation of the impact of hyperbilirubinemia, 20 mg bilirubin was dissolved in 1 ml of 0.1 M NaOH obtaining a stock solution of 20 g/L. Then, 40 µl of the stock solution was added to 960 µl of a serum sample to obtain a bilirubin level of 800 mg/L.

Acceptance criterion was that % bias between control and test sample due to interferences should be < TEa for bile acids (20%).

II. Method comparison

Patient samples were initially evaluated with 3rd generation method run routinely in the central laboratory and then categorized to one of the 3 bile acid concentration levels. The remainder sample was then divided in 3 aliquots and frozen at -80°C until analysis in batch, whereby it was assessed with the Immuno AU10V and the 3rd and 5th generation reagent method on the Pentra 400 analyzer.

The results obtained with the Immuno AU10V were compared with 2 methods of bile acid measurement run on a large bench top analyzer (ABX Pentra 400, ABX Horiba, Axonlab, Montpellier) serving as methods for comparison. The methods used for comparison included reagents of the 3rd (i.e., using an enzymatic-colorimetric method with NBT as dye, Bile acids, DiaSys Diagnostic Systems GmbH, Holzheim, Germany; subsequently called Pentra Diasys) and 5th (i.e., using the enzymatic-fluorimetric method with a 3α hydroxysteroid-dehydrogenase, Bile acids liquid, Labor+Technik, Berlin, Germany; subsequently called Pentra LT) generation, respectively. They were run on the same analyzer to rule out an

impact of the analyzer itself on the results. The 5th generation method run on the large bench top analyzer was considered as reference method.

Statistical analysis

Statistical software programs (MedCalc, software version 16.2.1; Ostend, Belgium and GraphPad Prism 6 Software, GraphPad Software, Inc., La Jolla, USA) were applied for statistical analysis.

Linearity and Recovery

Linearity for a feline sample spiked with serially diluted bovine bile acids under dilution was investigated for evaluation of the correlation between observed bile acid values plotted against a calculated (expected) bile acid concentration. The difference between actual and theoretical bile acid concentration was used to evaluate recovery after dilution:

$$\text{Recovery \%} = \frac{\text{measured concentration}}{\text{expected concentration}} * 100$$

The quality requirements for recovery after dilution was set at the range of 80 - 120% as recommended previously for validation of immunoassays (Andreasson et al. 2015). Correlation between expected and measured results was assessed with a linear and Deming regression analysis.

Precision and lower limit of quantification

Imprecision was calculated based on mean and standard deviation (SD):

$$\text{CV \%} = \frac{\text{SD}}{\text{Mean}} * 100$$

SD obtained in replication studies for assessment of precision should not exceed 0.25*TEa (20%). The respective cut of value was calculated as follows: [Mean of 10 replication measurements]/100*20*0.25.

Interferences

The mean %bias between test and control sample and thus the observed interference effect (d_{obs}) was determined as follows:

$$d_{obs}\% = \frac{mean_{test} - mean_{control}}{mean_{control}} * 100$$

D_{obs} % between control sample and sample spiked with interfering substances (hemoglobin, lipid, and bilirubin) should not exceed the TEa of 20%.

Method comparison

Correlation and bias between the methods was assessed with a Spearman´s rank analysis, Passing Bablock regression and a Bland Altman plot, respectively.

Correlations were considered "excellent" for Spearman´s rho (r_S) =0.93-0.99, "good" for r_S=0.80-0.92, "fair" for r_S=0.59-0.79, and "poor" for r_S< 0.59, respectively (Papasouliotis et al. 2006).

A Shapiro Wilk test was used to verify the assumption of normality. As non-normal distribution was present, a Kruskal-Wallis test was done to calculate the difference between median bile acid concentrations obtained for each analyzer and method.

To more objectively judge results, TEobs was assessed and compared with quality specification published previously for bile acids, i.e., the total allowable error (TEa) (Harr et al. 2013).

Quality requirements were fulfilled when TEobs < TEa (=20%).

TEobs = % bias +2*% coefficient of variation (CV)

Results:

I. Method validation

Ease of use

The Immuno AU10V was easy to use with a short training period.

For measurement, a cartridge, a tip for the pipette and the tube with the serum sample were inserted in the analyzer, an ID was entered for assignment of the sample and species was chosen by pressing the reference button. Cartridges had to be handled carefully without touching the surface. The analysis is started by pressing the start button. Calibrations and biochemical reactions were run automatically inside the cartridge. After 10 to 15 minutes the result was displayed on the screen.

At one study day, continuous use of the analyzer over 8 hours resulted in an error due to disturbed motor function probably caused by build-up of tips. However, the litter bins for tips was only three quarters filled and a remark to empty the litter bin had not appeared so far.

Linearity and recovery

The results of the two linearity experiments for feline pool plasma spiked with serially diluted bovine sodium hydrate cholate obtained for the 5th generation assay run on the large bench top analyzer and the immunoassay performed with Immuno AU10V respectively are shown in figure 1

A) and B).
As displayed in figure1, there was an excellent correlation between expected and measured results.

However, a marked bias ranging between 25-60% was seen for the Immuno AU10 V, when a sample spiked with bovine material was assessed. Similarly, recovery rate was ranging between 38% and 74% when specimens spiked with bovine bile acids were assessed (table 1).

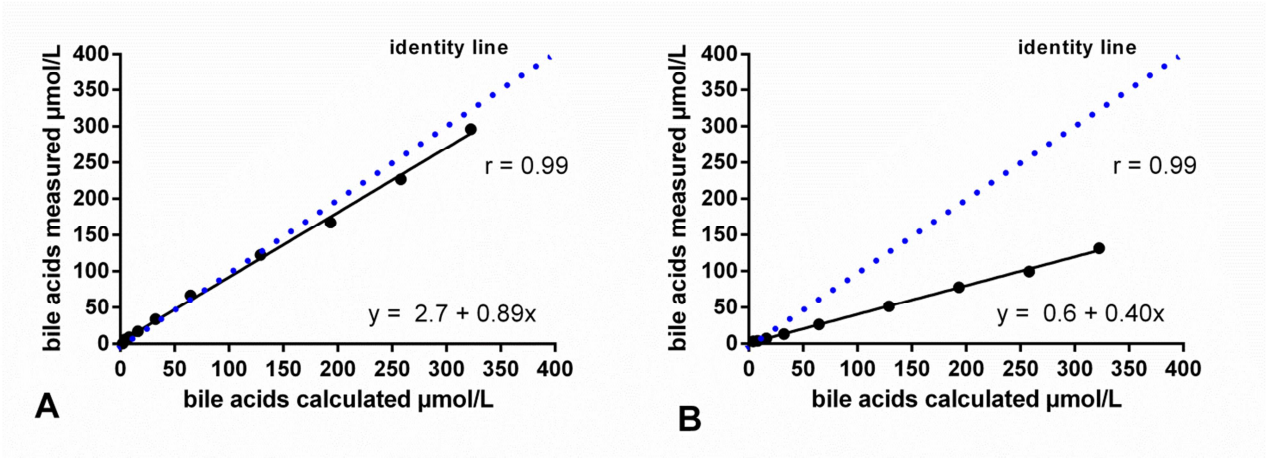

***Fig. 1**: Linearity of bile acid measurement of feline serum spiked with 10 µl of serially diluted bovine sodium hydrate cholate such that an initial concentration of 323 µmol/L was obtained*
(A) Linearity under dilution assessed with a 5th generation bile acid assay run on the Pentra 400 bench top analyzer (Penta-LT).
(B) Linearity under dilution assessed with the Immuno AU10 V.

Dilution Factor	Expected concentration [µmol/L]	Mean measured concentration [µmol/L]	Recovery [%]	Bias [%]	% bias < TE_a (20 %)
0.0125	4.03	3,00	**74.44**	**25.56**	**No**
0.025	8.06	3,9	**48.39**	**51.61**	**No**
0.05	16.13	7,23	**44.84**	**55.16**	**No**
0.1	32.26	13,1	**40.61**	**59.39**	**No**
0.2	64.52	26,37	**40.87**	**59.13**	**No**

0.4	129.03	51,47	**39.89**	**60.11**	**No**
0.6	193.55	77,77	**40.18**	**59.82**	**No**
0.8	258.06	99,67	**38.62**	**61.38**	**No**
1	322.58	131,37	**40.72**	**59.28**	**No**

Table 1*: Linearity and recovery rates of feline pool plasma spiked with 10 µl of serially diluted bovine bile acids to obtain samples containing bile acids in clinically relevant concentration ranges of 4 – 323 µmol/L.*
Recovery rates lower than 80% and TEobs exceeding the TEa of 20%, are marked in bold letters.

Abbreviations: *TEa = total allowable error, TEobs = total observed error*

Precision and lower limit of quantification

Intra- assay CVs calculated from 10 replicate measurements for canine and feline bile acids at 3 different concentration levels are shown in tables 2 and 3. As seen in the tables, intra- assay CV was mainly ≤ 3% for the dog and and ≤ 4% for the cat. In both species, quality requirements were fulfilled for all concentration levels. In the low concentration range, intra-assay CVs obtained with the POCA tended to be lower than those obtained with the large bench top analyzer. In the higher concentration ranges, CVs were similar or slightly higher than those obtained with the bench top analyzer.

three bile acid concentration ranges / analyzer	Dog*	Fuji (n=10 replicates)			Pentra Diasys (n=10 replicates)			Pentra LT (n=10 replicates)		
		mean	SD	CV	mean	SD	CV	mean	SD	CV
0-30 µmol/L	1	7.08	0.21	2.96	14.3	0.65	4.53	9.33	0.12	1.24
	2	6.07	0.12	1.91	11.2	0.35	3.10	7.26	0.32	4.37
31-80 µmol/L	3	62.3	0.81	1.31	67.5	0.57	0.84	63.6	0.59	0.92
	4	47.62	0.92	1.94	52.9	0.66	1.25	49.79	0.22	0.44
>80 µmol/L	5	123.26	2.23	1.81	129.76	0.57	0.44	112.11	0.77	0.68
	6	93.45	0.95	1.01	111.41	0.85	0.76	97.78	0.99	1.02

***Table 2**: Intra-assay CVs for the analysis of bile acids obtained from replicate measurements with the Immuno AU10V v-BA test (Fuji) and the two reference methods run on the large bench top analyzer Pentra 400.*

All results for SD were <0.25 TEa and thus fulfilled the quality criteria.

***Abbreviations:** SD= standard deviation, CV = coefficient of variation, TEa = total allowable error*

*****measurements obtained from a pool of dog serum with different bile acid concentration levels

three bile acid concentration ranges / analyzer	Cat*	Fuji (n=10 replicates)			Pentra Diasys (n=10 replicates)			Pentra LT (n=10 replicates)		
		mean	SD	CV	mean	SD	CV	mean	SD	CV
0-30 µmol/L	1	11.72	0.36	3.11	25.37	0.29	1.16	15.04	0.17	1.14
	2	8.21	0.14	1.77	17.8	0.38	2.15	9.99	0.16	1.60
31-80 µmol/L	3	65.35	0.84	1.28	85.43	0.45	0.53	75.23	0.69	0.96
	4	62.84	1.94	3.08	70.7	0.42	0.59	65.56	0.65	0.99
>80 µmol/L	5	108.12	4.20	3.88	127.53	0.81	0.64	118.02	0.91	0.77
	6	94.3	3.09	3,27	110.27	0.66	0.60	101.33	0.83	0.82

***Table 3**: Intra-assay CVs for the analysis of bile acids obtained from replicate measurements with the Immuno AU10V v-BA test (Fuji) and the two reference methods run on the large bench top analyzer Pentra 400.*

All results for SD were <0.25 TEa and thus fulfilled the quality criteria.

***Abbreviations:** SD= standard deviation, CV = coefficient of variation, TEa = total allowable error*

*****measurements obtained from a pool of cat serum with different bile acid concentration levels

For dogs, serum bile acids < 3.8 µmol/L were assessed with a sufficient quality, so that the LloQ of 2 µmol/L reported by the manufacturer could be confirmed (table 4).

Two low bile acid concentrations	Dog*	Intra-assay CV (n=20 replicates)			Quality criterion 0.25*TEa (µmol/L)	SD < 0.25*TEa
		Mean (µmol/L)	SD (µmol/L)	CV (%)		
≈ 4 µmol/L	1	3.77	0.14	3.83	0.19	Yes
≈ 6 µmol/L	2	6.15	0.14	2.21	0.31	Yes

Table 4*: Determination of the lower limit of quantification for canine serum: Intra-assay CVs for the bile acid analysis obtained from replicate measurements at low bile acid values with* the Immuno AU10V analyzer.

Abbreviations: *SD= standard deviation, CV = coefficient of variation, TEa = total allowable error*

*****measurements obtained from a pool of dog serum with different bile acid concentration levels

Interferences

As seen in tables 5 and 6, hemoglobin did not interfere with the bile acid measurement at concentrations up to 4g/L. In contrast, measurement of bile acids in canine samples spiked with 8g/L soy bean was not possible and false high bile acid concentrations were detected when canine samples were spiked with 4 g/L soy bean oil. In feline samples containing the same concentration of soy bean oil, measurement of bile acids was not possible, so that specimens with 2g/L soy bean oil had to be prepared in which bile acids could be measured with a bias of approximately 17% still fulfilling quality criteria. In canine and feline specimens spiked with 800 mg/L bilirubin, falsely high bile acid concentrations were detected.

Interferent	Mean bile acids $_{control}$ [µmol/L] ± SD	Mean bile acids $_{test}$ [µmol/L ± SD	Mean bias [µmol/L]	% bias	% bias < TE_a (20 %)
Hemoglobin 4 g/l	5.73 ± 0.29	5.8 ± 0.16	0.27	4.6	Yes
Soy bean emulsion 4 g/l	3.7 ± 0.05	4.6 ± 0.21	0.97	26.3	**No**
Bilirubin 800 mg/l	5.7 ± 0.00	7.2 ± 0.12	1.47	25.7	**No**

***Table 5**: Observed interference effects of bilirubin, hemoglobin and lipid on bile acid measurement in pooled dog serum samples with the Immuno AU10V.*
Test specimens (bile acids $_{test}$) spiked with the potentially interfering substances were compared to control samples (bile acids $_{control}$) spiked with an equal volume of the diluent used for preparation of the respective stock solution of the interfering substance applied to the test sample. Measurement of all specimens were performed in triplicates in random order. %Bias for the interfering substance was considered acceptable if %bias < total allowable error TE_a.

Interferent	Mean bile acids $_{control}$ [µmol/L] ± SD	Mean bile acids $_{test}$ [µmol/L] ± SD	Mean bias [µmol/L]	% bias	% bias < TE_a (20 %)
Hemoglobin 4 g/L	5.7 ± 0.12	5.8 ± 0.08	0.2	3.5	Yes
Soy bean emulsion 2 g/L	4.9 ± 0.12	5.8 ± 0.12	0.83	16.9	Yes
Bilirubin 800 mg/L	5.7 ± 0.12	7.4 ± 0.46	1.71	29.6	**No**

***Table 6**: Observed interference effects of bilirubin, hemoglobin and lipid on bile acid measurement in pooled cat serum samples assessed with the Immuno AU10V.*
For remainder key see table 5. In the lipemic samples containing 4g/L triglycerides, measurement of bile acids was not possible so that a sample containing 2 g/L triglycerides was prepared. %Bias for the interfering substance was considered acceptable if %bias < total allowable error TE_a.

II. Method comparison

Overall, 64 canine and 30 feline samples were included.

Results of the method comparison study are shown in figures 2 und 3. For both dogs and cats, there was an excellent correlation between the methods ranging between 0.97 and 0.99 in dogs and 0.94 to 0.97 in cats, respectively. While an absolute mean bias close to zero was present when the Immuno AU 10 V was compared to the 5th generation assay run on the Pentra analyser (Pentra LT), mean absolute bias was markedly higher when the immunoassay run on the Immuno AU10V and the 5th generation test performed on the Pentra respectively, were compared to the 3rd generation assay. For cats and dogs, mean absolute bias was ranging between 7-10 µmol/L and 12-14 µmol/L, respectively. Overall, mean bile acid concentrations detected with the 3rd generation assay were higher than those assessed with the 5th generation assay and the Immuno AU10V assay.

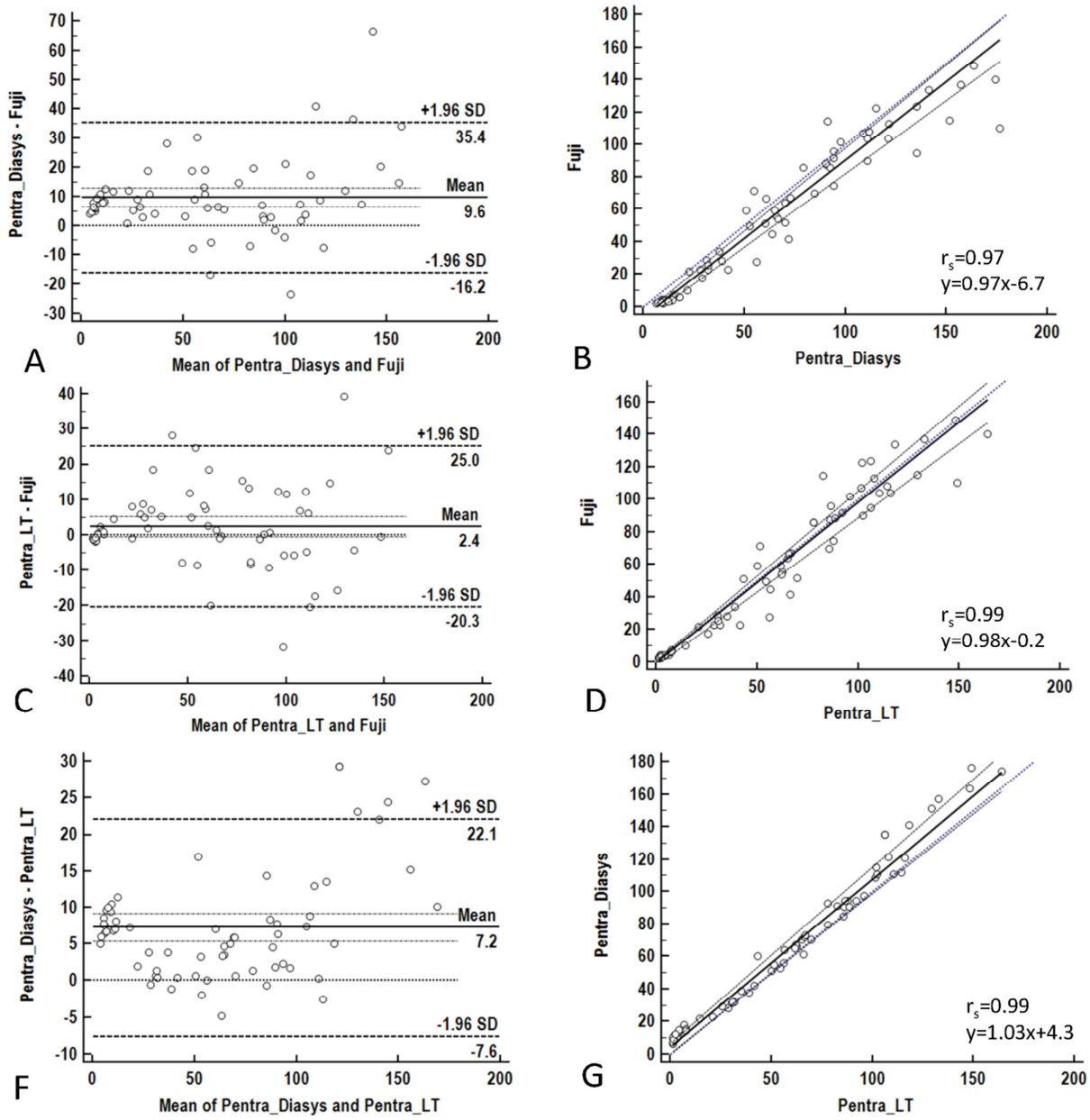

***Fig. 2**: Results of method comparison between the Immuno AU10V and a 3rd (Pentra Diasys) and 5th generation (Pentra LT) bile acid assay run on the automated analyzer ABX Pentra 400 (n=64 dog serum samples).*

Left: Bland-Altman difference plot demonstrating mean absolute bias (bold black line) with its 95% confidence interval (small black dotted line) und its 1.96fold standard deviation (SD) indicative of its limits of agreement (bold black dotted line).

Right: Passing- Bablok regression line (black line) with 90% confidence interval (dotted black line) of bile acid results assessed with the three methods. The identity line is indicated in blue.

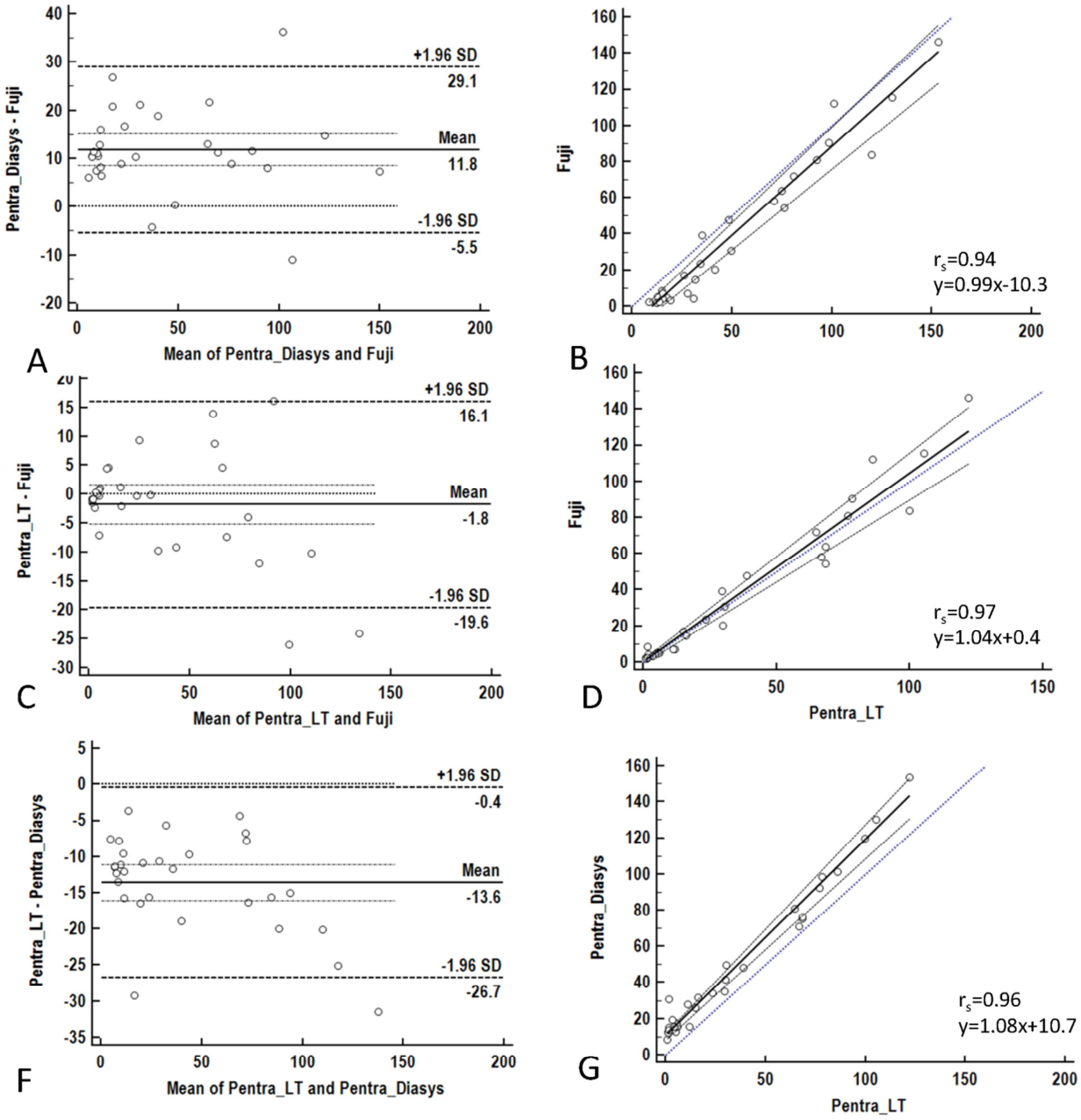

Fig. 3: *Results of method comparison between the Immuno AU10V and a 3rd (Pentra Diasys) and 5th generation (Pentra LT) bile acid assay run on the automated analyzer ABX Pentra 400 (n=30 cat serum samples).*

For remainder of key, refer to Fig. 2.

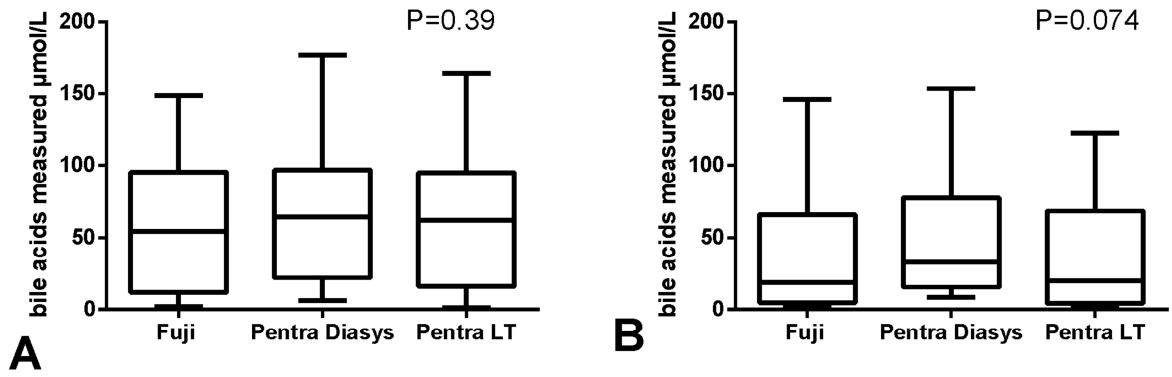

Fig. 4*: Box- and - whisker diagram demonstrating median and range of the bile acid measurements obtained with the three methods in dogs (n=64) and cats (n=30). The horizontal line in the boxes is consistent with the median, the whiskers indicate the range and the box represents the 25th -75th percentile.*

Nevertheless, statistical analysis did not reveal a significant difference between mean bile acid concentrations obtained with all methods and analyzers (Figure 4).

As shown in tables 7 and 8, quality requirements were fulfilled for canine and feline specimens when both the immunologic and the 5th generation assay were compared. Due to the large % bias > 30% when the 3rd generation bile acid assay was compared to the assay run on the Immuno AU10V and 5th generation bile acid performed on the Pentra 400, respectively TE_{obs} was > TE_a irrespectively of the analyzer.

three bile acid concentration ranges / analyzer	Dog*	Fuji vs. Pentra Diasys (n=10 replicates) Mean bias: 35.3%			Fuji vs. Pentra LT (n=10 replicates) Mean bias: 1.3%			Pentra Diasys vs. Pentra LT (n=10 replicates) Mean bias: 32.3%		
		mean	CV	TE_{obs} %	mean	CV	TE_{obs} %	mean	CV	TE_{obs} %
0-30 µmol/L	1	7.08	2.96	**41.2**	7.08	2.96	7.2	14.3	4.5	**41.4**
	2	6.07	1.91	**39.1**	6.07	1.91	5.1	11.2	3.1	**38.5**
31-80 µmol/L	3	62.3	1.31	**37.9**	62.3	1.31	3.9	67.5	0.84	**34.0**

	4	47.62	1.94	**39.2**	47.62	1.94	5.2	52.9	1.25	**34.8**
>80 µmol/L	5	123.26	1.81	**38.9**	123.26	1.81	6.2	129.8	0.44	**33.2**
	6	93.45	1.01	**37.3**	93.45	1.01	3.3	111.4	0.76	**33.8**

Table 7*: Total observed error (TE_{obs}) for bile acid test run on the Immuno AU10V when compared to a 3rd generation bile acid test run on the Pentra bench top analyser (Pentra Diasys) and a 5th generation performed on the Pentra analyzer (Pentra LT).*
TE_{obs} > TE_a (i.e. 20 %), i.e., not fulfilling quality requirements is shown in bold letters

Abbreviations*: vs = versus, TE_a = total allowable error, TE_{obs} = total observed error*

*****measurements obtained from a pool of dog serum with different bile acid concentration levels

three bile acid concentration range / analyzer	Cat*	Fuji vs. Pentra Diasys (n=10 replicates) Mean bias: 63,1%			Fuji vs. Pentra LT (n=10 replicates) Mean bias: 11,9%			Pentra Diasys vs. Pentra LT (n=10 replicates) Mean bias: 69%		
		mean	CV	TE_{obs} %	mean	CV	TE_{obs} %	mean	CV	TE_{obs} %
0-30 µmol/L	1	11.72	3.11	**69,3**	11.72	3.11	18.1	25.37	1.16	**71.3**
	2	8.21	1.77	**66,6**	8.21	1.77	15.4	17.8	2.15	**73.3**
31-80 µmol/L	3	65.35	1.28	**65,7**	65.35	1.28	14.5	85.43	0.53	**70.1**
	4	62.84	3.08	**69,3**	62.84	3.08	18.1	70.7	0.59	**70.2**
>80 µmol/L	5	108.12	3.88	**70,9**	108.12	3.88	19.7	127.53	0.64	**70.3**
	6	94.3	3.27	**69,6**	94.3	3.27	18.45	110.27	0.60	**70.19**

Table 8*: Total observed error (TE_{obs}) for bile acid test run on the Immuno AU10V when compared to a 3rd generation bile acid test run on the Pentra bench top analyser (Pentra Diasys) and a 5th generation performed on the Pentra analyzer (Pentra LT).*
TE_{obs} > TE_a (i.e. 20 %), i.e., not fulfilling quality requirements is shown in bold letters

Abbreviations*: vs = versus, TE_a = total allowable error, TE_{obs} = total observed error*

*****measurements obtained from a pool of cat serum with different bile acid concentration levels

Discussion

Overall, the results of the study demonstrated that the Immuno AU10V -vBA assay was easy to use and fulfilled all quality requirements when compared to a 5th generation assay run on a large bench top analyzer. When comparing both, the 3rd generation bile acid assay and the 5th generation test, a marked bias was present exceeding quality requirements for bile acids in veterinary medicine (Harr et al. 2013). The bias was also seen when both tests were run on the large bench top analyzer so that it is rather induced by the method than by the analyzer itself.

Regarding the linearity experiment, a marked bias ranging between 25-60% was seen for the Immuno AU10V, when a sample spiked with bovine material was assessed. The fact that the bias was only observed, when bovine bile acids were assessed but not when canine and feline specimen were evaluated indicates the species-specificity of the antibody used for Immuno AU10V -vBA assay.

For all methods and analyzers, intra-assay CVs fulfilled quality requirements. Interestingly, intra-assay CV of the Immuno AU10V tended to be lower in the low concentration range while for the tests run on the bench top analyzer, the contrary was observed. Generally, for laboratory methods, a higher CV is observed for the low concentration range rather than the higher concentration range. The relatively high intra-assay precision of the Immuno AU10V in the low concentration range might be attributed to the novel method of SPF used in the assays which is characterized by a high analytical sensitivity (Li et al. 2017).

Interference revealed false high results induced by hyperbilirubinemia at a concentration of 800 mg/l and lipemia at concentrations > 2 g/l. The results are in accordance with the effects described by the manufacturer of the 5th generation test for people. However, interfering effects of triglycerides were reported at higher concentrations in people, i.e., 7.5 g/L. The difference might be due to the method and the species. Moreover, different quality criteria for the definition of significant effect of the interfering substance also have an impact of the results, however, were not given by the respective manufacturer of the test.

Conclusion

Overall, the Immuno AU10V was an easy-to-use POCA which was capable to detect canine and feline bile acids with high precision and accuracy when compared to the 5th generation assay. Publication of the results appears worthwhile to facilitate promotion of the analyzer on the European and American market.

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References

Andreasson, Ulf; Perret-Liaudet, Armand; van Waalwijk van Doorn, Linda J. C.; Blennow, Kaj; Chiasserini, Davide; Engelborghs, Sebastiaan et al. (2015): A Practical Guide to Immunoassay Method Validation. In: *Frontiers in Neurology* 6. DOI: 10.3389/fneur.2015.00179.

Harr, Kendal E.; Flatland, Bente; Nabity, Mary; Freeman, Kathleen P. (2013): ASVCP guidelines: allowable total error guidelines for biochemistry. In: *Veterinary clinical pathology* 42 (4), S. 424–436. DOI: 10.1111/vcp.12101.

Li, Jian-Feng; Li, Chao-Yu; Aroca, Ricardo F. (2017): Plasmon-enhanced fluorescence spectroscopy. In: *Chemical Society reviews* 46 (13), S. 3962–3979. DOI: 10.1039/c7cs00169j.

Papasouliotis, Kostas; Cue, Simon; Crawford, Elsa; Pinches, Mark; Dumont, Michel; Burley, Ken (2006): Comparison of white blood cell differential percentages determined by the in-house LaserCyte hematology analyzer and a manual method. In: *Veterinary clinical pathology* 35 (3), S. 295–302.

Seibert, Rachel L.; Tobias, Karen M.; Reed, Ann; Snyder, Karl R. (2014): Evaluation of a semiquantitative SNAP test for measurement of bile acids in dogs. In: *PeerJ* 2. DOI: 10.7717/peerj.539.

Tisdall, P. L.; Hunt, G. B.; Tsoukalas, G.; Malik, R. (1995): Post-prandial serum bile acid concentrations and ammonia tolerance in Maltese dogs with and without hepatic vascular anomalies. In: *Australian veterinary journal* 72 (4), S. 121–126.