Validation of the particle-enhanced turbidimetric assay for measurement of feline serum and urinary cystatin C

In this document, the analytical validation of the particle-enhanced tubidimetric assay (PETIA) will be described.

Materials and methods

1. Cross reactivity

Because this assay is based on antibody-antigen reaction, it was mandatory to demonstrate cross reactivity between feline CysC and the anti-human CysC antibody of the assay by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Sample volumes of 20 µL serum and urine of two CKD (one proteinuric and one non-proteinuric) and one healthy cat were prepared. The serum and urine proteins were separated on a 18% resolving and 4.5% stacking SDS-PAGE gel with a colorimetric marker[a] and a human CysC standard[b]. After gel electrophoresis, the separated proteins were blotted on a nitrocellulose membrane with a transfer buffer containing 10% methanol. The residual binding sites were blocked with Tris buffered saline containing 1% Tween-20 (TBS-T) and 5% milk powder. The membrane was then incubated overnight at 2-8°C with the polyclonal anti-human rabbit CysC PETIA antibody[c] at 1/5000 dilution. After washing with TBS-T containing 5% milk powder, the blots were incubated for 1 h at room temperature with donkey anti-rabbit IgG Horseradish Peroxidase linked whole antibody[a] at a dilution 1/3000. Proteins were visualized with a chemiluminescent substrate[d] and exposed to a film.[d]

2. Assay sensitivity

Samples were analysed with PETIA using the Cobas auto-analyzer. Assay sensitivity was calculated based on the mean and corresponding standard deviation (SD) of the assay diluent (blank sample) analysis to determine the lowest concentration of sCysC and uCysC that can be measured.[1] According to Jensen et al.,[1] the limit of detection (LOD) was calculated as two times the SD above the mean blank sample value which was obtained from 20 replicate measurements.

3. Imprecision

Serum and urine samples of six CKD and six healthy cats were analyzed in duplicate on the same day and on three consecutive days. The intra-assay coefficient of variation (CV) was determined by dividing the SD of parallel measurements on the same day by their mean and then multiplied by 100.[1] The inter-assay CV was determined likewise from the measurement on three consecutive days.[1]

4. Linearity

The method accuracy was assessed by evaluation of the linearity under dilution.[1] For this purpose, one serum and one urine sample with both a high CysC concentration were serially diluted fourfold. The observed sCysC and uCysC concentrations were plotted against the expected concentrations and linear regression analysis was performed to calculate the corresponding CV.

Results

Cross reactivity

On Western blot analysis of the feline serum samples, a band was visible at the expected molecular mass (MM) of 13 kDa (Supplemental Figure 1). However, additional bands were visible at approximately 26 kDa, 52 kDa and higher MM. This was also seen at the human CysC standard (Supplemental Figure 1A). For urine samples, a band was again and now more clearly visible at the expected MM of 13 kDa, and was more pronounced in the cat with CKD and proteinuria (CKD/P). Tentatively, bands at the height of approximately 26 kDa and 52 were visible in the urine of cats with CKD, but not in the healthy cats (Supplemental Figure 1B).

Validation of the PETIA

The turbidimetric assay showed an excellent intra-assay CV for both serum and urine. A coefficient of variation of 15% is rather high, but considered satisfactory (Table 1).[1] PENIA however, showed a better inter-assay CV for urine. For serum, inter-assay CVs of both PENIA and PETIA are comparable. Additionally, the regression analysis after serial dilution of a serum and urine sample could not show a linear relationship between the observed and expected sCysC and uCysC concentration (Supplemental Figure 2 A and B).

Discussion

On Western immunoblotting with antibodies from PETIA, a band at the CysC MM of approximately 13 kDa was observed both in serum and urine (Fig 1), but the band was far weaker in serum compared with urine. Additional bands at the MM of 26 and 52 kDa were visible. These findings are comparable with the Western blot results from the PENIA, described in an earlier publication of our group.[2] The high MM bands have also been observed on Western immunoblotting with the same polyclonal anti-human CysC antibody in pooled serum samples from humans, dogs and cats.[3] The intra- and inter-assay CV for sCysC and the intra-assay CV for uCysC were comparable with the PENIA (Table 2), but the inter-assay CV for uCysC was higher than 15% which is still acceptable, but far less ideal than PENIA.[4] In addition, the LOD from the PETIA was high compared with the PENIA, and no linear relationship between the observed and expected sCysC and uCysC could be observed after serial dilution of a serum and urine sample. The sCysC concentration measured with the PETIA was also significantly higher than the PENIA, indicating that results of both immunoassays cannot be used interchangeably. Thus, based on the findings of the present study, we advise to measure feline CysC with the human PENIA, since a feline immunoassay is not available yet.

Acknowledgements

For this work support was received from the institute for the promotion of innovation by science and technology in Flanders (IWT) through a bursary to L.F.E. Ghys. A special thanks to Mr. Tom Nagels, Medic Lab Aalst, Belgium, for the use of the Cobas^{m} analyser for the validation of the PETIA; and to Libra Diagnostica for providing us the polyclonal anti-human rabbit CysC antibody from the turbidimetric assay.

End Notes

[a] Amersham Biosciences, GE Healthcare Europe GmbH, Diegem, Belgium

[b] Biorad, Nazareth-Eke, Belgium

[c] Dako, Glostrup, Denmark

[d] Perkin Elmer, Zaventem, Belgium

[e] particle enhanced turbidimetric assay, Dako, Glostrup, Denmark

[f] Cobas C system, Roche Diagnostics Gmbh, Mannheim, Germany

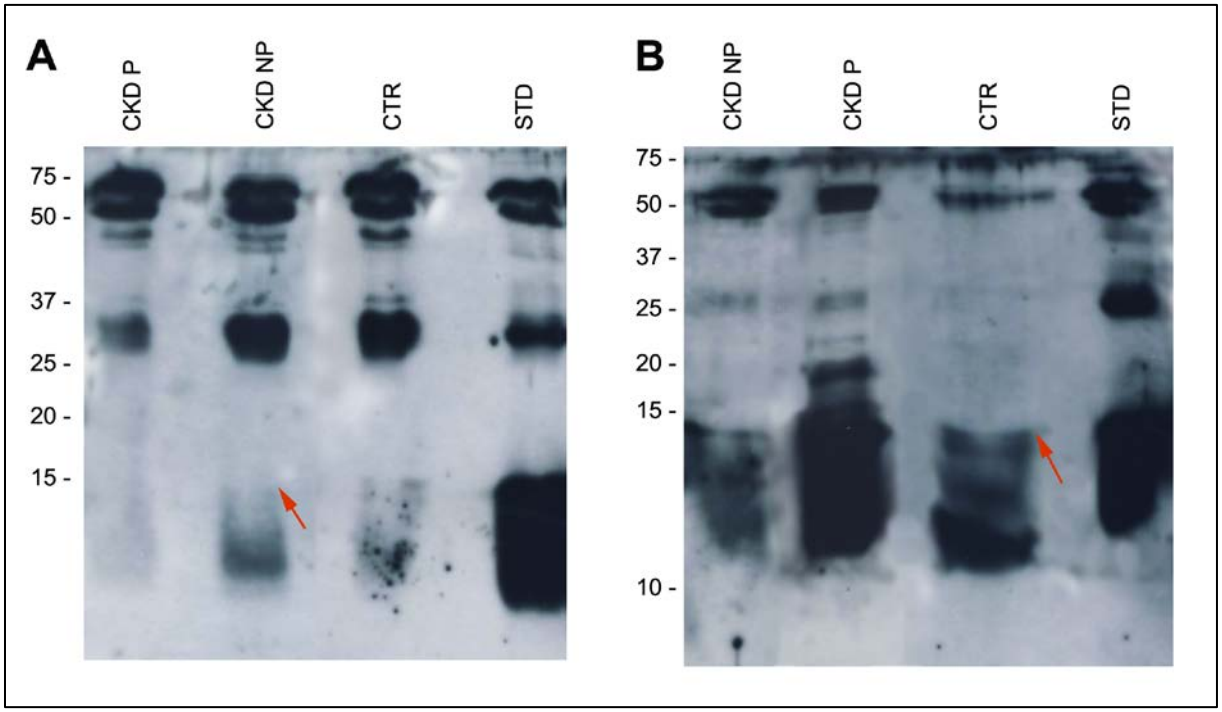
Table 1. Validation parameters of the turbidimetric immunoassay compared with the previously validated nephelometric assay (both n = 6 cats).[2]

	Validation parameter	PETIA	PENIA[2]
	Sensitivity		
	Limit of detection (mg/L)	0.39 mg/L	0.049 mg/L
Serum	*Precision*		
	Intra-assay coefficient of variation (%)	0.8	1.3
	Inter-assay coefficient of variation (%)	9.5	12.5
Urine	*Precision*		
	Intra-assay coefficient of variation (%)	0.4	0.4
	Inter-assay coefficient of variation (%)	15.9	4.1

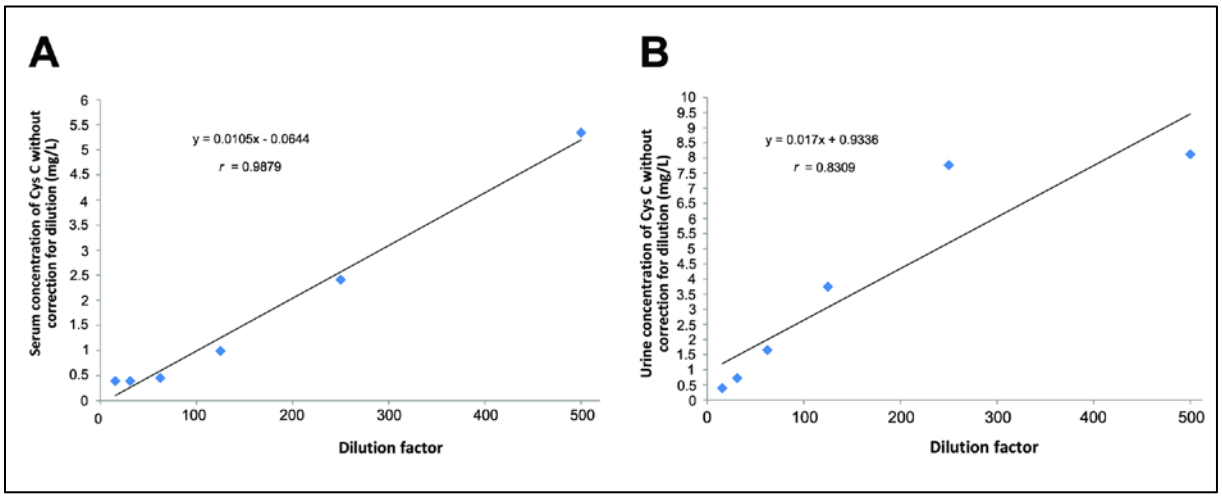
PETIA, particle enhanced turbidimetric immuno assay; PENIA, particle enhance nephelometric immunoassay.

Supplemental Figures

Supplemental Figure 1. Western Blot analysis with chemiluminiscent detection of the polyclonal rabbit anti-human cystatin C antibody from the particle enhanced turbidimetric immunoassay (PETIA) in feline serum **(1A)** and urine (**1B**). indicates 13 kDa molecular mass; numbers indicate the molecular mass (kDa); STD, human purified Cystatin C; M, colorimetric marker; H, healthy cat; CKD/NP, non-proteinuric cat with chronic kidney disease; CKD/P, cat with chronic kidney disease and proteinuria



Supplemental Figure 2. Sequential dilution of serum **(2A)** and urine **(2B)** of a cat with chronic kidney disease (CKD) analyzed with PETIA illustrating linearity. Cys C, Cystatin C; *r*, correlation coefficient.



References

1. Jensen AL. Validation of diagnostic tests in hematology laboratories. In: Feldman BV, Zinkl, F.G., Jain, N.C., ed. Schalm's Veterinary Hematology, 5th ed. Philadelphia: Lippincott Williams and Wilkins; 2000:20-28.
2. Ghys LFE, Meyer E, Paepe D, et al. Analytical validation of a human particle-enhanced nephelometric assay for cystatin C measurement in feline serum and urine. Vet Clin Pathol 2014;43:226-234.
3. Almy FS, Christopher MM, King DP, et al. Evaluation of cystatin C as an endogenous marker of glomerular filtration rate in dogs. J Vet Intern Med 2002;16:45-51.
4. Shah VP, Midha KK, Findlay JW, et al. Bioanalytical method validation-a revisit with a decade of progress. Pharm Res 2000;17:1551-1557.