Development of monoclonal antibodies and simplified sandwich ELISA for canine neutrophil gelatinase-associated lipocalin A detection

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Abstract

Background

Acute kidney injury (AKI) is one of the most common canine diseases with a high mortality rate. Neutrophil gelatinase-associated lipocalin (NGAL) is the novel biomarker for early diagnosis of renal injury. Only few sandwich ELISA kits are commercially available, all of which require the use of expensive enzyme-conjugated secondary or biotinylated antibody. The aims of this study were to develop high affinity monoclonal antibodies (mAbs) and simplified sandwich ELISA for canine NGAL detection.

Results

Recombinant canine NGAL was expressed in E. coli and purified to a high purity. Six hybridoma cell lines were generated by immunization of mice with the purified protein, all of which secreted high titers of specific mAbs. By screening 36 different antibody combinations, a pair of mAbs with high additivity and P/N ratio was selected as the capture and detection antibodies. By conjugation of the detection mAb with horse radish peroxidase, a simplified sandwich ELISA was developed with a correlation coefficient of 0.9939, detection limit of 8.28 ng/mL. The parallel test of 42 samples from healthy and AKI dogs showed the good agreement in NGAL concentrations detected by the simplified sandwich ELISA and commercial ELISA kit.

Conclusions

We developed canine NGAL-specific monoclonal antibodies and simplified sandwich ELISA. The simplified sandwich ELISA could replace the commercial ELISA kits for canine NGAL detection with the advantages of reduced cost and detection time.

Background

Acute kidney injury (AKI) is characterized by a sudden and sustained decrease in renal function. The disease is common in dogs with high mortality and treatment cost [1, 2]. One of the main reasons for the high mortality is late recognition of disease and consequently a narrow window of opportunity for intervention [3]. Currently, canine AKI is diagnosed mainly according to elevated concentration of serum creatinine and blood urea nitrogen [4]. However, early stages of AKI may not be detected when kidney function is assessed using traditional diagnostic tests, such as serum creatinine concentration [3].

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25-kDa glycoprotein synthesized in low concentrations in renal tubular, intestinal, hepatic and pulmonary tissues, which is substantially up-regulated with tissue injury [5]. Circulating NGAL is filtered by the glomerulus, reabsorbed in the proximal tubule and secreted by the thick ascending limb of the loop of henle. In active renal tubular injury, NGAL synthesis is increased, reabsorption is decreased and secretion is increased, resulting in increased urinary

NGAL (uNGAL) concentrations [6]. Therefore, NGAL has been used as one of the earliest and most accurate biomarkers for human and canine AKI diagnosis [7, 8].

Both serum NGAL (sNGAL) and uNGAL can be detected using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) [8–10]. However, only few ELISA kits are commercially available and their detection sensitivities may need further validation. In addition, these ELISA kits require the use of expensive enzyme-conjugated secondary antibodies or biotinylation of mAbs [11]. In the present study, we expressed recombinant canine NGAL in E. coli and generated six hybridoma cell lines by immunization of mice with the purified protein. By systemic screening, a pair of mAbs with high advitivity was selected as the antibody combination for sandwich ELISA. By conjugation of the detection mAb, a simplified sandwich ELISA was established for canine NGAL detection.

Results

Preparation of recombinant canine NGAL

After induction with IPTG, SDS-PAGE analysis showed that an expected 26-kDa protein (25 kDa NGAL + 0.6 kDa His-tag) was expressed in pET-NGAL recombinant E. coli (Fig. 1). The recombinant NGAL was expressed as a soluble protein and purified to more than 90% purity by using nickel affinity column (Fig. 1). Commercial canine NGAL ELISA analysis showed that the purified protein reacted positively to canine NGAL antibody.

Generation of hybridoma cell lines

After three immunizations, the three immunized mice had serum ELISA antibody titers of 1:32000, 1:128000 and 1:16000, respectively. The spenocytes were isolated from the mouse with the highest antibody titer for myeloma cell fusion. After HAT selection and dilution cloning, 10 positive cell clones were screened by indirect ELISA using recombinant canine NGAL. Subsequent to two rounds of subcloning, six stable hybridoma cell lines, namely NC-1, NC-2, NC-3, NC-4, NC-5 and NC-6, were obtained for further study.

Characterization of canine NGAL-specific mAbs

The specific mAbs were prepared in large quantities by peritoneal injection of mice with different hybridoma cell lines. Western blotting analysis showed that the six mAbs reacted positively with the canine NGAL reference (Fig. 2). Isotype ELISA analysis showed that the six mAbs belonged to IgG2a, IgG1, IgG1, IgG2b, IgG2b and IgG1 isotypes, respectively (Table 1), with the antibody titers ranged from 1:1024000 to 1:8192000 (Fig. 3). The affinity test showed that the six mAbs had the Ka values ranged from 4.14×10^7 to 1.52×10^8 (Table 1), which could be ranked as NC-2 > NC-3 > NC-1 > NC-4 > NC-5 > NC-6.

Table 1
Characterization of monoclonal antibodies against canine NGAL protein

	NC-1	NC-2	NC-3	NC-4	NC-5	NC-6
IgG subtype	IgG2a	IgG1	IgG1	IgG2b	IgG2b	IgG1
Ka value	$9.86 \cdot 10^7$	$1.52 \cdot 10^8$	$1.02 \cdot 10^8$	$6.32 \cdot 10^7$	$4.14 \cdot 10^7$	$9.28 \cdot 10^6$

Screening of the antibody pair for simplified sandwich ELISA

The additivity assay showed that, among 36 mAb combinations screened, 16 mAb combinations had the AI values ≥ 50% (Table 2). These antibody combinations were further screened by checkerboard titration and the best combination was selected according to the highest P/N ratio. The result showed that NC-5/NC-3 combination had the highest P/N ratio (Fig. 4). Therefore, NC-5 and NC-3 mAbs were selected as the capture and detection antibodies for the sandwich ELISA.

Table 2
ELISA additivity test for binding of mAbs to NAGL.

mAb		Capture antibody					
		NC-1	NC-2	NC-3	NC-4	NC-5	NC-6
Detection antibody	NC-1	0	86.92%	3.20%	73.7%	81.39%	71.41%
	NC-2	85.52%	0	59.35%	5.57%	28.57%	2.05%
	NC-3	2.80%	63.82%	0	79.24%	83.28%	74.69%
	NC-4	72.40%	8.28%	88.03%	0	17.73%	6.05%
	NC-5	83.22%	19.06%	80.87%	12.24%	0	15.50%
	NC-6	72.21	0.44%	77.66%	4.02%	10.09%	0

Optimization of simplified sandwich ELISA

By using different mAb concentrations, checkerboard titration showed that the P/N ratios increased as the increase of NC-5 and NC-3 mAb concentrations. Based on to the highest P/N ratio, the optimal concentrations of NC-5 and NC-3 mAbs were 2 μg/mL and 1:8000, respectively. To optimize the coating conditions, ELISA plates were coated with NC-5 mAb in three different buffers for different times at 4 °C. The result showed that the highest P/N ratio could be obtained by coating with NC-5 mAb in 50 mM Tris-HCl (pH 8.0) for 45 min (Fig. 6a and 6b). To optimize the blocking conditions, the antibody-coated plates were blocked with two different blocking solutions for three different times at 37 °C. The result showed that the highest P/N ratio could be obtained by blocking with 1% casein in PBST for 3 h (Fig. 6c). To

optimize the dilution buffer and incubation time for the detection antibody, NC-3 mAb was diluted with PBST containing 1% casein or 5% BSA and incubated for different times at 37 °C. The result showed that the highest P/N ratio could be obtained by incubation with NC-3 mAb in PBST containing 1% casein for 45 min (Fig. 6d).

Generation of standard curve for simplified sandwich ELISA

The double-antibody sandwich ELISA was performed under optimal conditions using different concentrations of recombinant canine NGAL. The standard curve was generated by plotting OD_{450} values against NGAL concentrations. As shown in Fig. 7, the standard curve was linear with NGAL concentration (log10) ranging from 6.25 ng/mL to 1600 ng/mL, with a correlation coefficient of 0.9939, detection limit of 8.28 ng/mL.

Reproducibility of simplified sandwich ELISA

The reproducibility assays showed that the double-antibody sandwich ELISA had an intra-CV ranging from 5.2–8.7% (n = 20) and inter-CV ranging from 8.7–9.6% (n = 10).

Validation of simplified sandwich ELISA

A total of 42 clinical samples were detected in parallel using the simplified sandwich ELISA and a commercial canine NGAL kit. Among 21 samples from healthy dogs, the concentrations of sNGAL detected by the simplified sandwich ELISA ranged from 9.75 ng/mL to 17.34 ng/mL, which were slightly lower than that detected by commercial ELISA kit (Table 3). The concentrations of uNGAL detected by the simplified sandwich ELISA ranged from 7.86 ng/mL to 24.38 ng/mL with a variation range slightly narrower than that detected by commercial ELISA kit. Among 21 samples from AKI patients, both concentrations of sNGAL and uNGAL detected by the simplified sandwich ELISA were slightly lower than that detected by commercial ELISA kit. Further analysis of 21 samples from AKI dogs showed that both concentrations of sNGAL and uNGAL detected by two different ELISA were linear without significant difference (Fig. 8).

Table 3
Comparison of detection performance between in-home and commercial ELISA

	Health samples		AKI samples (ng/mL)	
	Serum (ng/mL)	Urine (ng/mL)	Serum (ng/mL)	Urine (ng/mL)
In-home ELISA	9.15–21.40	9.21–28.75	86.89-266.72	103.97-336.87
Commercial ELISA	14.66–73.76	4.00-63.06	119.64-306.33	117.67-340.16

Discussion

NGAL is the novel biomarker for reliable diagnosis of renal failures in humans and dogs [12, 13]. However, the concentrations of NGAL in different samples from different patients vary greatly [3] and thus ambiguous diagnosis may be obtained by using different NGAL ELISA kits [14–17]. In addition, the currently available canine NGAL ELISA kits require the use of expensive enzyme-conjugated secondary antibodies or biotinylation of mAbs [11]. To alleviate these problems, in this study we expressed canine NGAL in E. coli as a His-tag fusion protein. Most eukaryotic proteins over-expressed in E. coli are insoluble inclusion bodies, which require complicated denaturation/renaturation process to obtain correctly folded products. This was also the case for recombinant canine NGAL expressed in E. coli at 37 ℃. Since higher temperature is one of main contributors to the formation of inclusion bodies, the expression of recombinant canine NGAL was induced at lower temperature (15 ℃) and the expected soluble product was obtained, which was purified to more than 90% purity without the need of denaturation and renaturation.

Although few mAb-based canine NGAL ELISA kits are commercially available, the detailed information about their preparation and characterization are not available [12]. In this study, we generated hybridoma cell lines by immunization of mice with highly purified recombinant canine NGAL. Among ten positive clones screened, six stable cell lines were obtained after HAT selection and repeated dilution cloning. All of the six mAbs reacted positively to canine NGAL reference with high titers and binding affinities, suggesting their suitability for the development of diagnostic reagents.

In this study, two key parameters were used to select the antibody combination for the simplified sandwich ELISA, including high AI value and P/N ratio. Among 36 antibody combinations screened, 16 of them had high (≥ 50%) AI values for canine NGAL, among which NC-5/NC-3 was selected for sandwich ELISA development since its highest P/N ratio. After systemic protocol optimization, the standard curve was generated for the simplified sandwich ELISA, with good linear with NGAL concentrations, high correlation coefficient and relatively high recovery rate.

The parallel detection results of clinical samples from healthy and AKI dogs showed that both uNGAL and sNGAL concentrations detected by the simplified sandwich ELISA were slightly lower than that detected by commercial ELISA kit. Nevertheless, the detection results of the two ELISA were linear without significant difference. These data suggest that the simplified sandwich ELISA established in this study can replace the commercial ELISA kits for canine NGAL detection. By using the simplified sandwich ELISA, both detection cost and time could be reduced significantly.

Conclusions

We expressed canine NGAL in E. coli and generated six stable hybridoma cell lines by immunization of mice with the purified protein. The specific mAbs obtained are useful for canine NGAL study and the simplified sandwich ELISA established can be used for the diagnosis of dog renal failures.

Methods

Preparation of recombinant canine NGAL

The coding sequence for canine NGAL (GenBank accession no. 491320) was adapted to E. coli codon usage using Java Codon Adaption Tool [18]. The synthetic sequence was cloned into pET28a (+) vector (Addgene, Shanghai, China) as an NdeI/BamHI fragment. The coding sequence was expressed as a C-terminally His-tagged protein under the control of T7 promoter. The recombinant vector was transformed into E. coli strain BL21 (DE3) and the expression of recombinant protein was induced overnight with 0.2 mM IPTG at 15 ℃. The bacterial cells were collected by centrifugation for 10 min at 5,000 g, suspended in PBS (1/10 culture volume), and disrupted two times at 1300 bar using a High Pressure Cell Disruptor (JNBIO, Guangzhou, China). After centrifugation for 10 min at 12,000 g, the recombinant protein was purified from the clarified cell lysate using Ni-NTA Sefinose Column (Sangon Biotech, China) and Hitrap QFF Anion Exchange Column (GE Healthcare, USA). The 6 × His tag was removed by digestion with thrombin protease (Sangon Biotech, China) by following the manufacturer's instruction. The identity of the purified protein was determined using commercial canine NGAL ELISA Kit (Sinobest Biotech Ltd., Shanghai, China).

Generation of hybridoma cell lines

Hybridoma cell lines were generated as previously described [19, 20]. Three female BALB/c mice (6–8 weeks old) were purchased from and housed in a single cage in the SPF-free facilite of Experimental Animal Center, Nantong University. Each mouse was immunized intramuscularly with recombinant canine NGAL (50 μg/50 μL) emulsified in 50 μL of Freund's complete adjuvant (Sigma, USA). The mice were immunized two more times with the same amount of the antigen emulsified in Freund's incomplete adjuvant at 2-week intervals. After three immunizations, mice were boosted intravenously with 100 μg of recombinant canine NGAL without adjuvant. The immunized mice were anaesthetized by carbon dioxide and sacrificed by cervical dislocation at day 4 post boosting immunization. The splenocytes were collected and fused with SP2/0 myeloma cells at a ratio of 10:1 in the presence of polyethylene glycol. The fused cells were suspended in HAT (hypoxanthine, aminopterin and thymidine) medium and plated onto 96-well plates. Four days later, HAT medium was replaced with HT medium. The supernatants of hybridoma cells were screened by indirect ELISA using recombinant canine NGAL as the antigen. The identity of recombinant canine NGAL was confirmed using commercial canine NGAL ELISA Kit by following the manufacturer's instruction. The positive hybridoma cells were cloned two times by dilution cloning for further study.

Characterization of canine NGAL-specific mAbs

To prepare the mAbs in large quantities, 10 female BALB/c mice (8–9 weeks old) were pre-injected with 0.5 mL of paraffin oil (Sigma-Aldrich, USA) followed by intraperitoneal injection with hybridoma cells ($5 \cdot 10^6$ cells/0.2 mL). The ascites were collected one week later and the mAbs used as the capture and detection antibodies of simplified sandwich ELISA were purified by ammonium sulfate precipitation as previously described [21].

The isotypes of mAbs were determined using Mouse mAb Isotyping Kit (Sigma-Aldrich, USA) by following the manufacturer's instruction. The mAbs were identified by Western blotting using canine NGAL reference. Briefly, canine NGAL was purified from dog urine samples with AKI as previously described [22]. The purified protein (50 μg) was separated by SDS-PAGE and transferred onto PVDF membrane. After blocking with 5% defatted milk powder in PBST (PBS containing 0.05% Tween 20) for 2 h at room temperature, the ascetic mAbs (1:5000 in blocking buffer) were added and incubated overnight at 4 ℃. After three-time washing in PBST, HRP-conjugated goat anti-mouse IgG (1:1000 in blocking buffer;Beyotime Biotechnology, China) was added and incubated as described. After washing again, the hybridization signals were developed using BeyoECL Plus Kit (Beyotime Technology, China) by following the manufacturer's instruction.

The ascitic mAbs were titrated by indirect ELISA using recombinant canine NGAL as the antigen. Briefly, 96-well plates were coated overnight with the recombinant antigen (2 ∝g/mL) and blocked with PBS containing 5% BSA for 2 h at room temperature. Serially diluted mAbs were added (100 μL/well) and incubated for 1 h at room temperature. After wash three times with PBST (PBS and 0.5% Tween 20), HRP-conjugated goat anti-mouse IgG (1:2000; Beyotime, China) was added (100 μL/well) and incubated again as described. After washing three times again, freshly prepared tetramethylbenzidine (TMB) solution was added and incubated in dark for 10 min at 37 ℃. OD_{450} values were determined for P/N value calculation after stopping with 2 M H_2SO_4. The mAb affinities for canine NGAL was tested by ELISA as previously described. The Ka value was calculated according to the formula: Ka = (A0-Ai) / Ii · Ai [23].

Selection of antibody pair for simplified sandwich ELISA

The additivity of each mAb pair for canine NGAL was tested by competing ELISA as previously described [24]. The additive index (AI) was defined for a pair of mAbs according to the formula: $AI = \{[2 \cdot A_{1+2}/(A_1 + A_2)\ ]\text{-}1\} \cdot 100$, where A_1, A_2 and A_{1+2} indicate the OD values of the first mAb, second mAb and two mAbs together in the ELISA. Provided the concentrations of mAbs are saturated for the antigen, the AI will tend to be zero if both mAbs recognize the same epitope, but close to 100 if both mAbs bind to distinct sites. The mAb pairs with high AI values were further tested by checkerboard ELISA and the best antibody combination was selected according to the highest P/N ratio.

Establishment of simplified sandwich ELISA

The simplified sandwich ELISA was established according to previously described method [8].The detection antibody was labeled with a 1:2 mAb to HRP (Thermo, USA) ratio according to the manufacturer's instruction. To optimize the concentrations of the antibody pair, ELISA was performed with different concentrations of NC-5 mAb as the capture antibody and different dilutions of HRP-conjugated NC-3 mAb as the detection antibody. To optimize the capture antibody coating buffer, ELISA plates were coated with NC-5 mAb in carbonate buffer (pH 9.6), Tris-HCl buffer (pH 8.0) or PBS (pH 7.4). To optimize the blocking buffer, the antibody-coated plates were blocked with PBST (0.01% Tween 20 in PBS) containing 1% casein or 5% BSA for 3 h at room temperature. To optimize the antigen reaction time, the antigen-added plates were incubated for different times at room temperature. To optimize the

incubation time for detection antibody, HRP-conjugated NC-3 mAb was diluted in PBST containing 1% casein or 5% BSA and incubated for different times at room temperature. Finally, ELISA plates were coated overnight with NC-5 mAb (2 μg/mL) at 4 ℃ and blocked with PBST containing 5% BSA for 2 h at room temperature. The recombinant canine NGAL or clinical samples was added and incubated for 45 min at room temperature. After washing three times with PBST, HRP-conjugated NC-3 mAb (1:8000) was added and incubated as described. After washing again, freshly prepared TMB solution was added and incubated in dark for 15 min. Subsequent to termination with 2 M H_2SO_4, OD_{450} values were measured on ELISA reader (Enspire 2300, PerkinElmer, USA).

Generation of standard curve for simplified sandwich ELISA

The standard curve for simplified sandwich ELISA was generated with different concentrations (0, 6.25, 12.5, 25, 50, 100, 200, 400, 800 and 1600 ng/mL) of recombinant canine NGAL using. Intra-assay coefficient (20 repeats) inter-assay coefficients (10 repeats) of variation values were determined using different concentrations of recombination canine NGAL (15 and 150 ng/mL)[25].

Validation of simplified sandwich ELISA

Both serum and urine samples were collected from healthy (n = 21) and AKI canine patients (n = 21) at Ai-Bi Pet Hospital and KangMei Pet Hospital (Nantong, China). The samples were detected in triplicates using the simplified sandwich ELISA and commercial canine NGAL ELISA kit (Sinobest Biotech, Shanghai, China) by following the manufacturer's instruction. The agreements between the concentrations detected by two different ELISA were submitted to linear regression analysis using Origin 8 software (OriginLab, Northampton, USA).

Abbreviations

AKI:Acute kidney injury; NGAL:Neutrophil gelatinase-associated lipocalin; mAb:monoclonal antibody; HRP:horse radish peroxidase; uNAGL:urinary NGAL; sNGAL:serum NGAL; ELISA:enzyme-linked immunosorbent assay; IPTG:isopropyl thiogalactoside; SDS-PAGE:sodium dodecyl sulfate polyacrylamide gel electrophoresis; AI value:additive index value; PVDF:Poly vinylidene fluoride; PBST:Phosphate Buffer Solution with Tween 20; TMB:tetramethylbenzidine.

Declarations

Ethics approval and consent to participate

Mouse immunization experiment was approved by the Experimental Animal Committee of the Nantong University (Approval No. 20190307-012) and conducted in accordance with the Guidelines for Experimental Animals of the Ministry of Science and Technology.

Consent for publication

Not applicable

Availability of data and materials

All data generated and/or analysed during the current study are included in this published article and its complementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JC involved in recombinant canine NGAL expression, hybridoma cell line generation and ELISA development. BT, XYZ and XLX performed canine NGAL detection. HCS polish the manuscript. All authors approved the final version of the manuscript.

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Not applicable

References

1. Cowgill LD, Langston CE. Acute kidney insufficiency. In: Bartges J, Polzin D, eds. Nephrology and Urology of Small Animals. Ames, IA: Blackwell Publishing Ltd; 2010:472–523.
2. Vaden SL, Levine J, Breitschwerdt EB. A retrospective case-control of acute renal failure in 99 dogs. J Vet Intern Med 1997; 11:58–64.
3. Palm CA, Segev G, Cowgill LD, LeRoy BE, Kowalkowski KL, Kanakubo K, Westropp JL. Urinary Neutrophil Gelatinase-associated Lipocalin as a Marker for Identification of Acute Kidney Injury and Recovery in Dogs

with Gentamicin-induced Nephrotoxicity. J Vet Intern Med. 2016; 30(1):200-5.

4. Thoen ME, Kerl ME. Characterization of acute kidney injury in hospitalized dogs and evaluation of a veterinary acute kidney injury staging system. J Vet Emerg Crit Car. 2011; 21(6):648-57.
5. Segev G, Palm C, LeRoy B, Cowgill LD, and Westropp JL. Evaluation of Neutrophil Gelatinase-Associated Lipocalin as a Marker of Kidney Injury in Dogs. J Vet Intern Med. 27 2013; 27:1362-7.
6. Soni SS, Cruz D, Bobek I, Chionh CY, Nalesso F, Lentini P, de Cal M, Corradi V, Virzi G, and Ronco C. NGAL: A Biomarker of Acute Kidney Injury and Other Systemic Conditions. Int urol nephrol. 2010; 42:141-50.
7. Koyner JL, Bennett MR, Worcester EM, Ma Q, Raman J, Jeevanandam V, Kasza KE, O'Connor MF, Konczal DJ, Trevino S, Devarajan P, and Murray PT. Urinary Cystatin C as an Early Biomarker of Acute Kidney Injury Following Adult Cardiothoracic Surgery. Kidney Int. 2008; 74:1059-69.
8. Lee YJ, Hu YY, Lin YS, Chang CT, Lin FY, Wong ML, Kuo-Hsuan H, Hsu WL. Urine neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute canine kidney injury. BMC Vet Res. 2012; 8:248.
9. Davis J, Raisis AL, Cianciolo RE, Miller DW, Shiel RE, Nabity MB, Hosgood GL. Urinary neutrophil gelatinase-associated lipocalin concentration changes after acute haemorrhage and colloid-mediated reperfusion in anaesthetized dogs. Vet Anaesth Analg. 2016; 43(3):262-70.
10. Cortellini S, Pelligand L, Syme H, Chang YM, Adamantos S. Neutrophil Gelatinase-Associated Lipocalin in Dogs With Sepsis Undergoing Emergency Laparotomy: A Prospective Case-Control Study. J Vet Intern Med. 2015; 29(6):1595-602.
11. Steinbach S, Weis J, Schweighauser A, Francey T, Neiger R. Plasma and Urine Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Dogs with Acute Kidney Injury or Chronic Kidney Disease. J Vet Intern Med. 2014; 28(2):264-69.
12. Monari E, Troìa R, Magna L, Gruarin M, Grisetti C, Fernandez M, Balboni A, Giunti M, and Dondi F. Urine Neutrophil Gelatinase-Associated Lipocalin to Diagnose and Characterize Acute Kidney Injury in Dogs. J Vet Intern Med. 2019; 1-10. DOI: 10.1111/jvim.15645
13. Cobrin AR, Blois SL, Abrams-Ogg ACG, Kruth SA, Dewey C, Holowaychuk MK, Gauthier V. Neutrophil gelatinase-associated lipocalin in dogs with chronic kidney disease, carcinoma, lymphoma and endotoxaemia. J Small Anim Pract. 2016; 57(6):291-98.
14. Zhou XB, Ma B, Lin Z, Qu Z, Huo Y, Wang JF, Li B. Evaluation of the usefulness of novel biomarkers for drug-induced acute kidney injury in beagle dogs. Toxicol Appl Pharm. 2014; 280(1):30-35.
15. Hsu WL, Lin YS, Hu YY, Wong ML, Lin FY, Lee YJ. Neutrophil Gelatinase-Associated Lipocalin in Dogs with Naturally Occurring Renal Diseases. J Vet Intern Med. 2014; 28(2):437-42.
16. Jung HB, Kang MH, Park HM. Evaluation of serum neutrophil gelatinase-associated lipocalin as a novel biomarker of cardiorenal syndrome in dogs. J Vet Diagn Invest. 2018; 30(3):386-91.

17. Hokamp JA, Cianciolo RE, Boggess M, Lees GE, Benali SL, Kovarsky M, Nabity MB. Correlation of Urine and Serum Biomarkers with Renal Damage and Survival in Dogs with Naturally Occurring Proteinuric Chronic Kidney Disease. J Vet Intern Med. 2016; 30(2):591-601.
18. Grote A, Hiller K, Scheer M, Münch R, Nörtemann B, Hempel DC, Jahn D. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res. 2005; 33:W526-31.
19. Sarikhani S, Mirshahi M, Gharaati MR, Mirshahi T. Generation of a novel high-affinity monoclonal antibody with conformational recognition epitope on human IgM. Appl Biochem Biotech. 2010; 162(5):1249-57.
20. Hadji-Ghasemi F, Gharagozlou S, Ghods R, Roohi A, Khoshnoodi J, and Shokri F. Generation and characterization of a mouse monoclonal antibody with specificity similar to staphylococcal protein A (SPA). Hybridoma Hybridom. 2003; 22(1):33-39.
21. Park S, Shukla S, Kim Y, Oh S, Hun KS, and Kim M. Development of Sandwich Enzyme-Linked Immunosorbent Assay for the Detection of Cronobacter Muytjensii (Formerly Called Enterobacter Sakazakii). Microbiol Immunol. 2012; 56:472-9
22. Xu JC, Li GY, Wang PL, Velazquez H, Yao XQ, Li YY, Wu YL, Peixoto A, Crowley S, Desir GV. Renalase is a novel, soluble monoamine oxidase that regulates cardiac function and blood pressure. J Clin Invest. 2005; 115:1275-80.
23. Bobrovnik SA. Determination of Antibody Affinity by ELISA. Theory. J Biochem Bioph Meth. 2003; 57:213-36.
24. Cao BL, Qiu HK, Hu JD, Meng FD, Zhang H, and Zhao HK. Characterization of Monoclonal Antibodies against Goat Interleukin-18 and Their Application in the Measurement of Goat Interleukin-18 in Lps-Stimulated Peripheral Blood Mononuclear Cells by Sandwich Vet Immunol Immunop. 2010; 138:235-8.
25. Thomsson O, Ström-Holst B, Sjunnesson Y, Bergqvist Validation of an enzyme-linked immunosorbent assay developed for measuring cortisol concentration in human saliva and serum for its applicability to analyze cortisol in pig saliva. Acta Vet Scand. 2014; 56:55. 54(12):647-55.

Figures

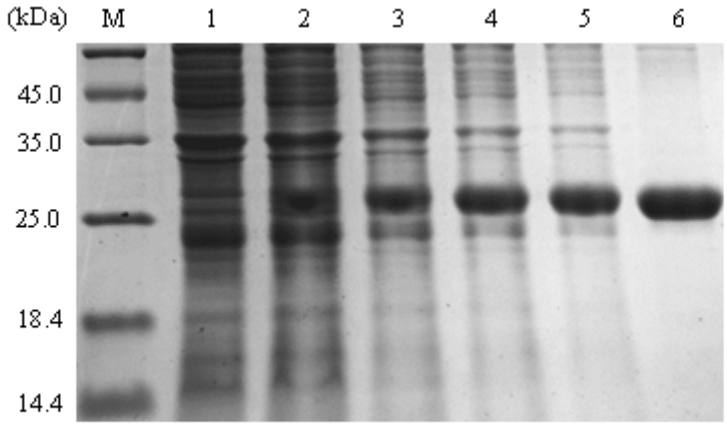

Fig. 1 Expression and purification of recombinant canine NGAL in *E. coli*

Figure 1

Expression and purification of recombinant canine NGAL in E. coli. The expression of recombinant canine NGAL was induced overnight with IPTG at 15 °C. The cell lysate before induction (1), IPTG-induced cell lysate (2), centrifuged cell lysate (3) and recombinant protein eluted with 50 (4), 100 (5) or 200 mM (6) imidazole were analyzed by 12% SDS-PAGE.

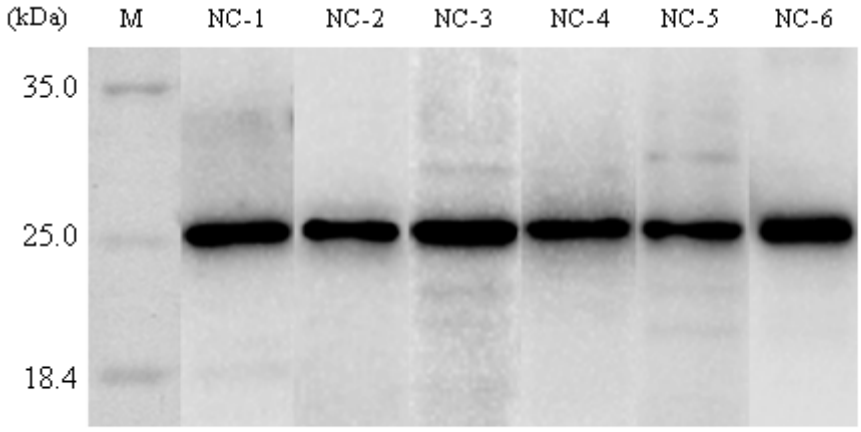

Fig. 2 Identification of canine NGAL-specific mAbs by Western blotting.

Figure 2

Identification of canine NGAL-specific mAbs by Western blotting. The canine NGAL was purified from the urine samples of AKI dogs and analyzed by Western blotting using mAbs from six hybridoma cell lines.

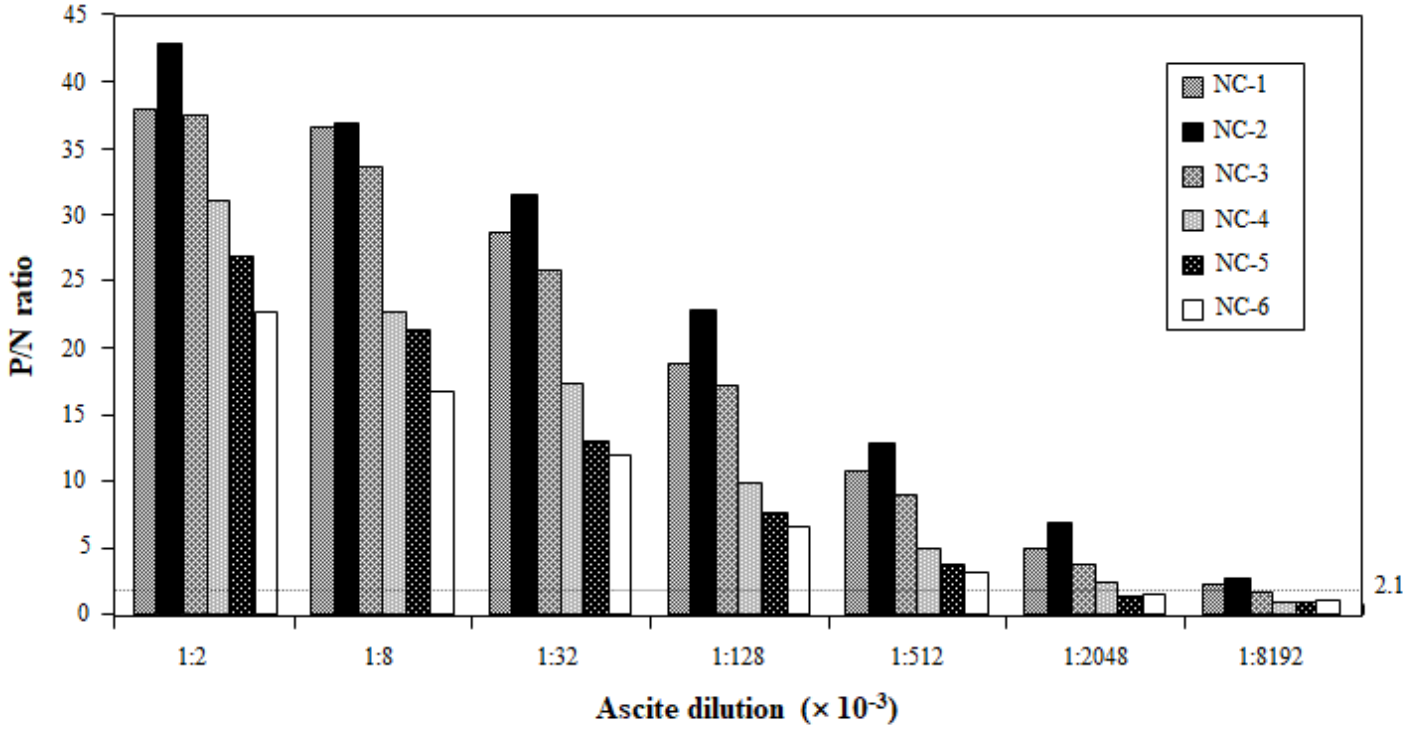

Fig. 3 Titration of canine NGAL-specific mAbs by indirect ELISA

Figure 3

Titration of canine NGAL-specific mAbs by indirect ELISA. Different dilutions of ascitic mAbs secreted by the six hybridoma cell lines were titrated against recombinant canine NGAL. The dotted line indicates the cutoff of P/N ratio.

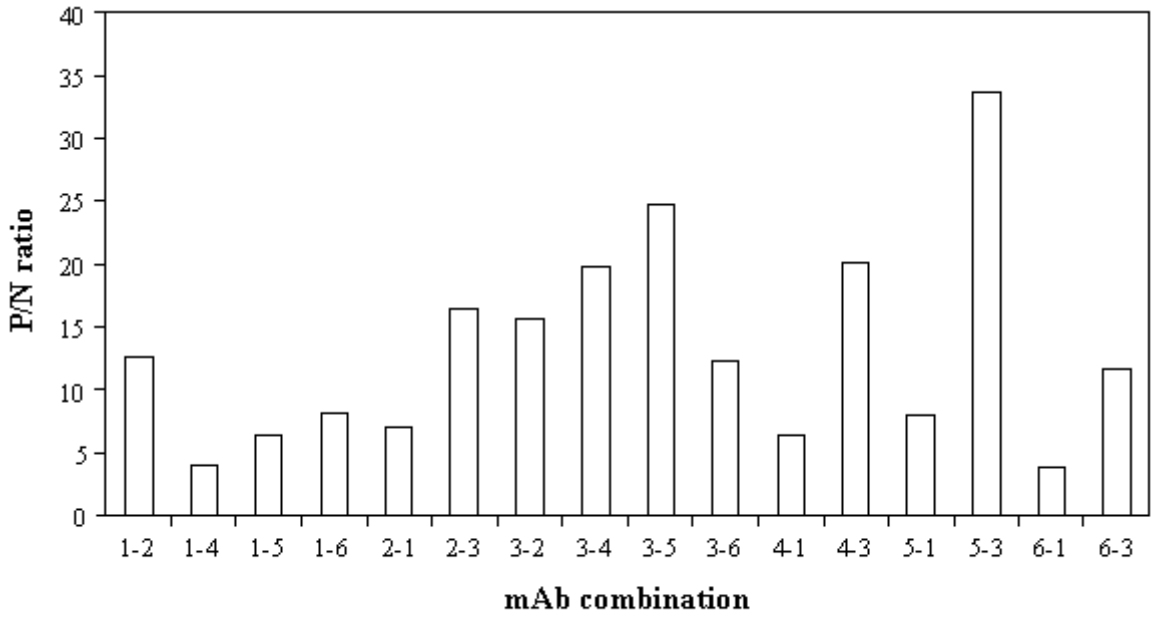

Fig. 4 Screening of the mAb combination for simplified sandwich ELISA

Figure 4

Screening of the mAb combination for simplified sandwich ELISA. Recombinant canine NGAL was reacted with different mAb combinations and their binding additivities were expressed P/N ratios.

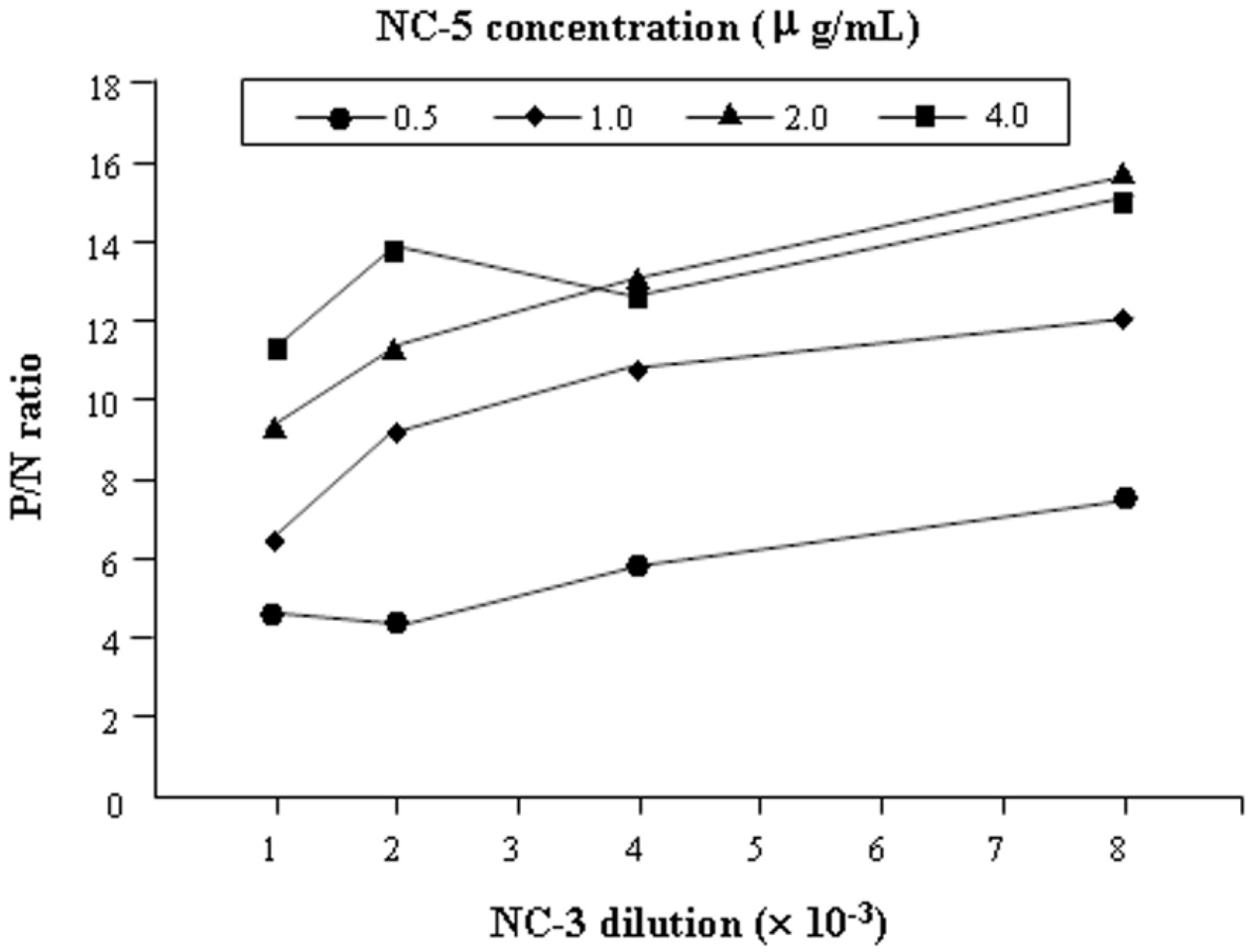

Fig. 5 Checkerboard titration of the antibody pair for simplified sandwich ELISA

Figure 5

Checkerboard titration of the antibody pair for simplified sandwich ELISA. ELISA was performed using the indicated concentrations of NC-5 mAb as the capture antibody and indicated dilutions of NC-3 mAb as the detection antibody. The best antibody concentrations were selected according to the highest P/N ratio.

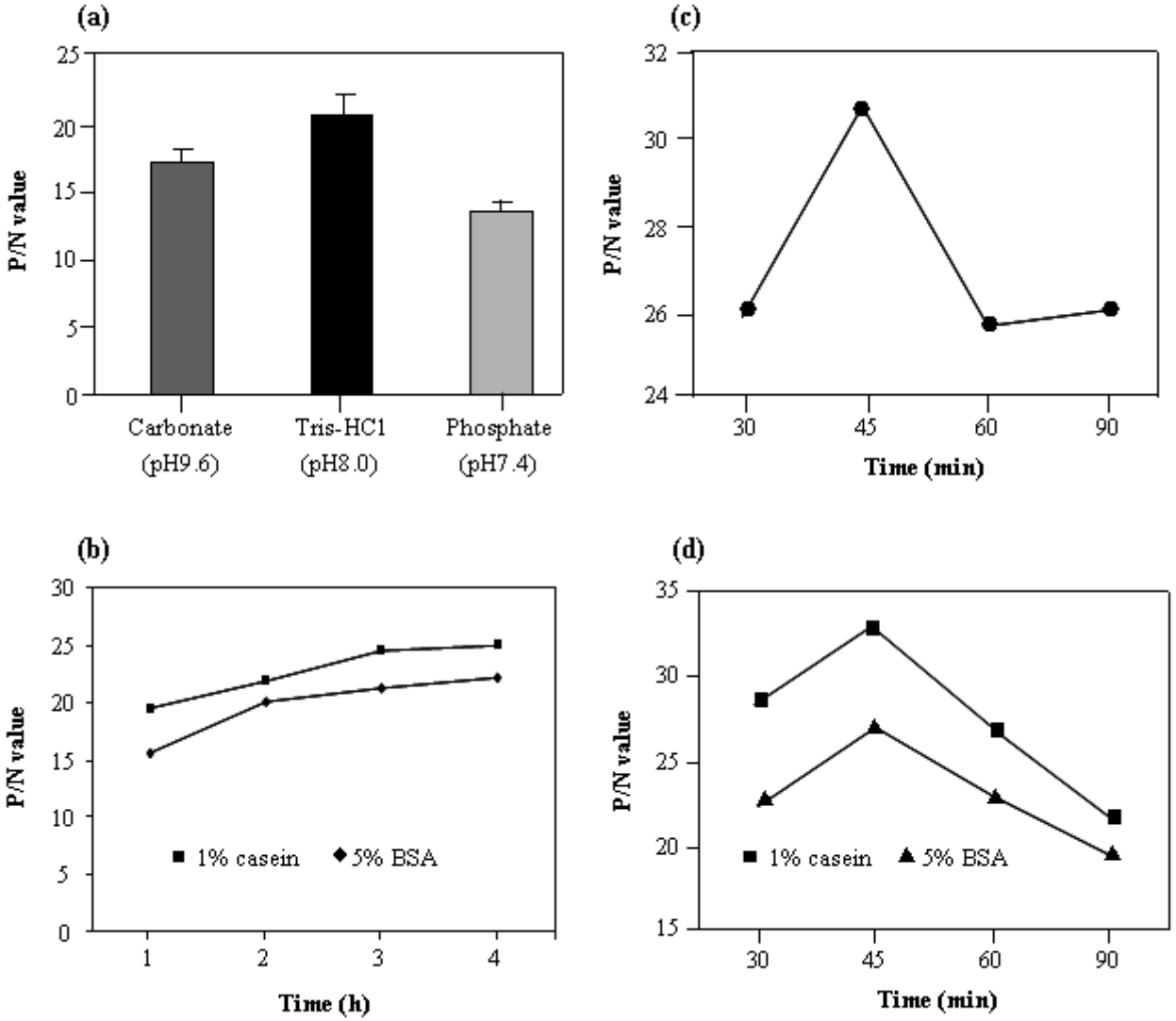

Fig. 6 Optimization of simplified sandwich ELISA for canine NGAL detection

Figure 6

Optimization of the simplified sandwich ELISA for canine NGAL detection. (a) ELISA plates were coated overnight with recombinant canine NGAL in three different buffers; (b) The antigen-coated plates were blocked with two different buffers for different times. (c) Clinical samples was added to the blocked plate and incubated for different times; (d) HRP-conjugated NC-3 mAb was diluted in two different buffers and incubated for different times. The optimal conditions were selected according to the highest P/N ratios.

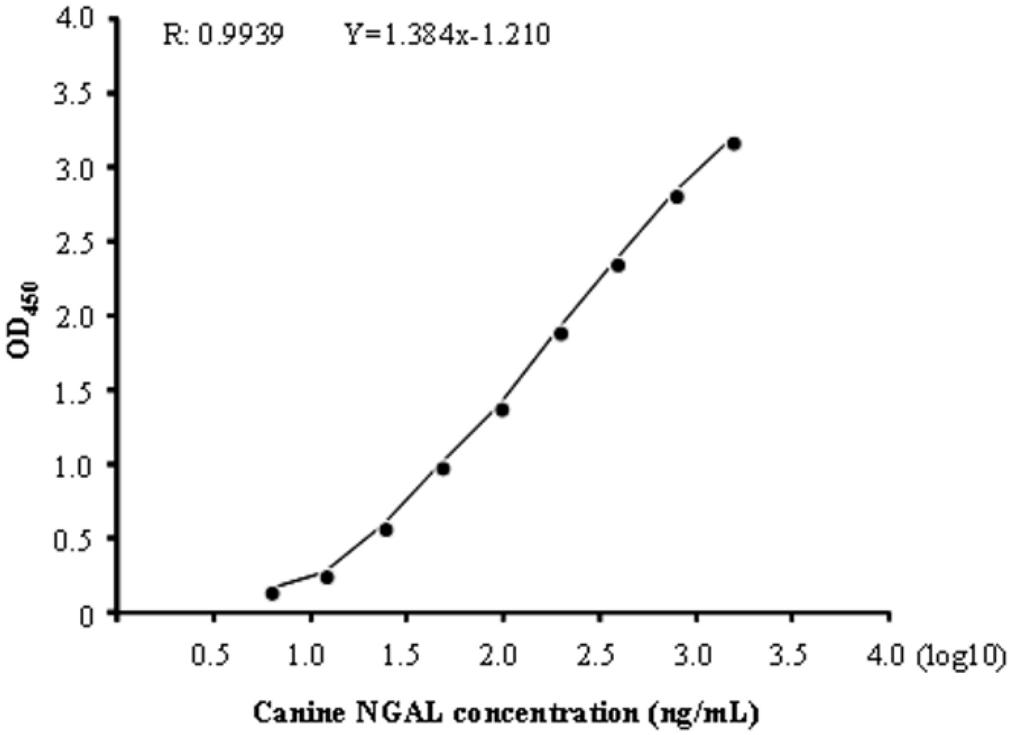

Fig. 7 The stand curve for simplified sandwich ELISA

Figure 7

The standard curve for simplified sandwich ELISA. The ELISA was performed with different concentrations of recombinant canine NGAL and the standard curve was plotted against OD450 values.

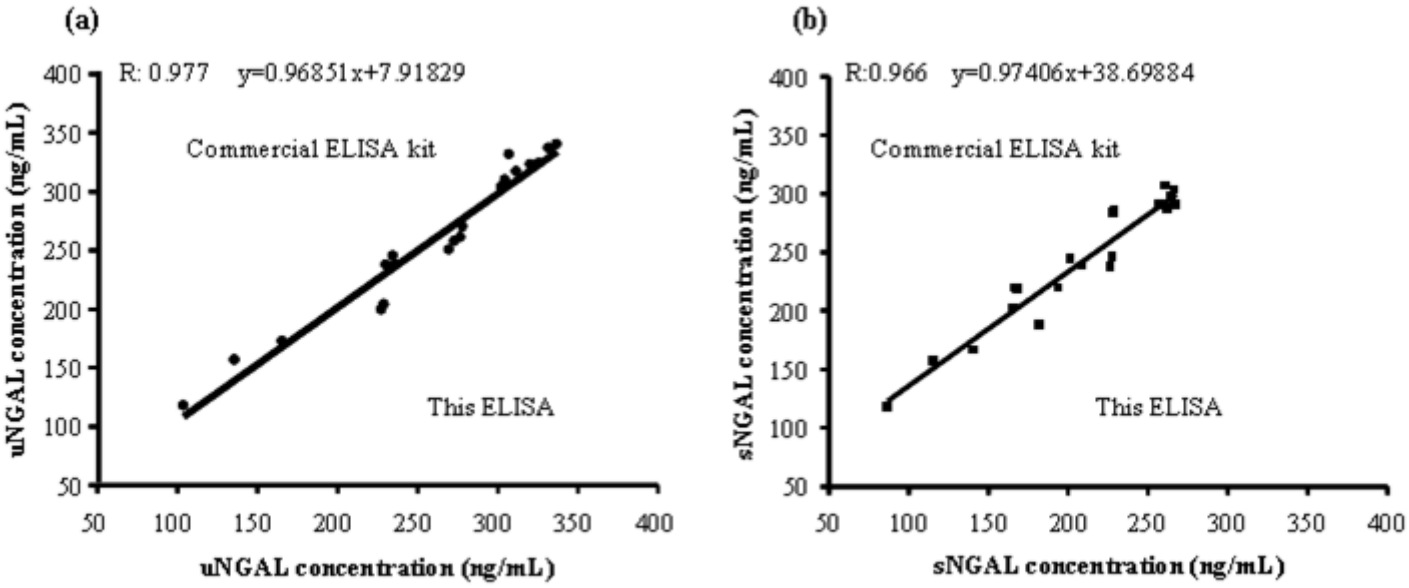

Fig. 8 Agreements of canine NGAL concentrations detected by two different ELISA

Figure 8

Agreements of canine NGAL concentrations detected by two different ELISA. Twenty one serum and urine samples from AKI dog patients were detected in parallel by the simplified sandwich ELISA and commercial ELISA kit and their linear agreements were analyzed using Origin 8 software.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.xls
- NC3RsARRIVEGuidelinesChecklist.docx
- Additionalfile2.xls