Analytical Biochemistry 457 (2014) 65–73

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Cold-microwave enhanced enzyme-linked immunosorbent assays—A path to high-throughput clinical diagnostics

Niels Grützner [a,*,1], Romy M. Heilmann [a,1], Jan S. Suchodolski [a], Jörg M. Steiner [a], Andreas Holzenburg [b,c,d]

[a] Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, Texas A&M University, College Station, TX 77843, USA
[b] Microscopy and Imaging Center, Texas A&M University, College Station, TX 77843, USA
[c] Department of Biology, Texas A&M University, College Station, TX 77843, USA
[d] Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843, USA

ARTICLE INFO

Article history:
Received 27 February 2014
Received in revised form 15 April 2014
Accepted 18 April 2014
Available online 26 April 2014

Keywords:
Calprotectin
Cold-microwave technology
ELISA
Feces
Serum
Validation

ABSTRACT

The enzyme-linked immunosorbent assay (ELISA) constitutes an important clinical diagnostic approach. However, the prolonged incubation times involved lead to turnaround times of typically ⩾1 day, potentially delaying a definitive diagnosis or an adequate treatment plan for individual patients. Here cold-microwave technology (CMT) was employed to significantly reduce the times required for diagnostic ELISAs. The new approach was validated and compared to a conventional ELISA setup measuring canine calprotectin (cCP). Canine serum and fecal specimens were used for the analytical validation of cCP ELISA by conventional and CMT–ELISA. Cross-validation of both ELISA methods consisted of the determination of analytic sensitivity, linearity, accuracy, precision, and reproducibility. The long-term stability of antibody-coated ELISA plates was also evaluated up to 33 days. The ELISA approaches were comparable to each other. The observed-to-expected ratios for linearity and accuracy were 100.2 ± 11.8 and 98.1 ± 10.8% (mean ± standard deviation), respectively. Precision and reproducibility were ⩽17.2%. For samples run on precoated ELISA plates over 33 days %CVs were ⩽12.5%. While both ELISA approaches were analytically sensitive, linear, accurate, precise, and reproducible with measurements of cCP concentrations, CMT–ELISA offered a reduction in incubation times by 90–95%, facilitating a very fast turnaround time and suggesting CMT–ELISA for improved human and veterinary clinical diagnostics.

Enzyme-linked immunosorbent assays (ELISAs)[2] are used to detect and quantify antigens and are widely used as a research and diagnostic tool in human and veterinary medicine.

Diagnostic laboratories use ELISAs to measure analytes diagnosing and/or monitoring specific pathological conditions, such as gastrointestinal diseases presented here. In canine patients, idiopathic inflammatory bowel disease (IBD) is a chronic inflammation of the small or large intestine, the exact cause of which is unknown [1]. Diagnosis of IBD in human and canine patients is challenging and requires comprehensive testing to exclude other causes of chronic gastrointestinal inflammation [2,3]. Thus, biomarkers of inflammation specific for the gastrointestinal tract appear to be clinically useful for the diagnosis and assessment of disease progression in patients with IBD.

Calprotectin, the S100A8/A9 (calgranulin A/B) protein complex, is expressed and released into the extracellular space by activated macrophages and neutrophils and can be induced in epithelial cells. Serum and fecal concentrations of calprotectin have been shown to be clinically useful as a marker of disease activity in human patients with IBD [4–6] and have also been shown to be increased in canine patients with gastrointestinal diseases such as IBD [7,8].

ELISAs are time-consuming because the incubation time associated with each individual ELISA step can take up to 1 h [9], leading to overall running times of an entire day for diagnostic ELISAs, potentially delaying adequate treatment for an individual patient. To reduce incubation time, cold-microwave technology (CMT) was employed as it has been previously shown to enhance the rate of antibody–antigen interaction in Western blot applications [10]. It was therefore speculated that CMT could be applied to ELISAs in a clinical diagnostic setting.

Therefore, the aim of this study was (1) to develop and analytically validate a novel ELISA approach by using CMT for quantifying serum and fecal canine calprotectin (cCP) concentrations and (2) to compare this CMT–ELISA to the conventional ELISA, hypothesizing

* Corresponding author. Fax: +1 (979) 458 4015.
E-mail address: tierarztbz@hotmail.com (N. Grützner).
[1] These authors contributed equally to this work.
[2] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; IBD, inflammatory bowel disease; CMT, cold-microwave technology; cCP, canine calprotectin; *O*/*E*, observed-to-expected.

http://dx.doi.org/10.1016/j.ab.2014.04.026
0003-2697/

that the CMT–ELISA compares favorably to the conventional ELISA in terms of turnaround time while maintaining the levels of precision and reliability.

Materials and methods

Sample population, collection, and processing of specimens

Eight serum and eight fecal samples from different canine individuals were obtained from an unrelated research project at the College of Veterinary Medicine at Texas A&M University. Serum samples were obtained from dogs by venipuncture of the jugular vein, and fecal samples were collected immediately after defecation. All serum and fecal specimens were stored in polypropylene tubes and kept frozen at −20 °C until use. The protocol for collection of serum and fecal samples had been reviewed and approved by the Clinical Research Review Committee at Texas A&M University (CRRC No. 2010-05).

Polyclonal antibody production

Polyclonal antibodies against purified cCP [11] were raised in New Zealand White rabbits (*Oryctolagus cuniculus*) by repeated inoculation with purified cCP emulsified in complete and incomplete Freund adjuvant [12]. Blood was drawn and antibody titers were evaluated in the sera [12]. Polyclonal anti-cCP antibodies were purified by affinity chromatography and conjugated with horseradish peroxidase (HRP; EZ-Link Plus activated peroxidase, Thermo Scientific, Waltham, MA, USA), followed by storage at −20 °C in a conjugate stabilizer solution (SuperFreeze conjugate stabilizer, Thermo Scientific).

Conventional ELISA approach

The conventional sandwich ELISA was conducted as described in Table 1. ELISA plates (Nunc-Immuno ELISA plate, Thermo Scientific) were coated with the affinity-purified polyclonal anti-cCP followed by blockage of nonspecific binding sites. Canine CP standard solutions (1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 µg/L), blanks, and three quality controls with different cCP concentrations were prepared in ELISA buffer (25 mM Tris/HCl, 150 mM NaCl, 0.05% Tween 20, 0.5% bovine serum albumin, pH 8.0) and were applied to each ELISA plate in duplicate fashion. Fecal samples were extracted according to Heilmann et al. [13]. Following antigen capture using an HRP-conjugated anti-cCP antibody, ELISA plates were developed with a stabilized 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (1-Step Ultra TMB ELISA, Thermo Scientific), the absorbance was measured using an automated plate reader (Synergy 2 Alpha microplate reader, BioTek, Winooski, VT, USA), and a commercial software package (Gen5 data analysis software (version 1.05), BioTek) was used to calculate a five-parameter logistic curve fit ($y = f[x] = d + [(a - d)/(1 + (x/c)^b)^e]$) and to determine cCP concentrations in test samples.

CMT–ELISA approach

CMT–ELISA was conducted using materials and reagents as described above; the ELISA protocol is summarized in Table 1. For each incubation step, a CMT with a ColdSpot (PELCO BioWave ColdSpot Pro laboratory microwave system, Ted Pella, Redding, CA, USA) unit was used, which allows for temperature control between 0 and 60 °C, ensuring consistent standardized conditions across the entire ELISA plate (Fig. 1). This allows for use of the CMT system for already existing ELISAs (i.e., conventional cCP ELISA) at the respective temperature settings. Furthermore, the CMT uses wattage control between 100 and 750 W allowing for a specific rate of molecular (antibody–antigen) interactions [14]. The following conditions were used for the CMT–ELISA: calibration power of 450 W at 37 °C with a 6-min cycle (2 min on–off–on) [14]. Samples were analyzed in the same fashion as described above.

In addition, long-term stability of antibody-coated ELISA plates stored at 4 °C and subjected to CMT–ELISA was determined. Briefly, 11 ELISA plates were coated with anti-cCP, blocked as described above, and stored at 4 °C until further use. Canine CP concentrations were evaluated in eight different samples (five serum samples and three fecal extracts) on days 1, 3, 5, 8, 12, 15, 19, 22, 26, 29, and 33, followed by evaluation of the effect of storage time

Table 1
Assay protocol for conventional ELISA and cold-microwave technology (CMT)–ELISA.

Assay step	Conventional ELISA	CMT–ELISA
(1) Primary Ab (100 µl each well)	150 ng α-cCP/well[a]	300 ng α-cCP/well[a]
Incubation at 37 °C	1 h[b]	6 min[c]
Wash step ×3 (TBS–Tween)[d]		
(2) Blocking (200 µl each well)	Blocking buffer[e]	Blocking buffer[e]
Incubation at 37 °C	1 h[b]	6 min[c]
Wash step ×3 (TBS–Tween)[d]		
(3) Loading (100 µl each well)	Standards and samples	Standards and samples
Incubation at 37 °C	1 h[b]	6 min[c]
Wash step ×3 (TBS–Tween)[d]		
(4) Secondary Ab (100 µl each well)	12 ng α-cCP/well[f]	12 ng α-cCP/well[f]
Incubation at 37 °C	1 h[b]	6 min[c]
Wash step ×3 (TBS–Tween)[d]		
(5) Substrate and development	TMB solution[g]	TMB solution[g]
	10 min (plate shaker)	20 min (bench top)
(6) ELISA stop solution	Stopping solution[h]	Stopping solution[h]
Total incubation time	4 h	24 min

All ELISA steps are shown for both the conventional ELISA and the CMT–ELISA. Superscript letters refer to materials and reagents that were used for the ELISAs.

[a] 200 mM carbonate–bicarbonate; Pierce, Rockford, IL, USA.
[b] Incubator, Boekel Scientific, Feasterville, PA, USA.
[c] PELCO BioWave ColdSpot Pro Laboratory Microwave System, Ted Pella, Redding, CA, USA.
[d] 25 mM Tris/HCl, 150 mM NaCl, 0.05% Tween 20; pH 8.0.
[e] 25 mM Tris/HCl, 150 mM NaCl, 0.05% Tween 20, 10% bovine serum albumin (BSA); pH 8.0.
[f] 25 mM Tris/HCl, 150 mM NaCl, 0.05% Tween 20, 0.5% BSA; pH 8.0.
[g] 1-Step Ultra TMB ELISA, Thermo Scientific, Waltham, MA, USA.
[h] 4 M acetic acid, 1 N sulfuric acid.