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Publication Date

2024-02-01

DOI

10.1016/j.jim.2023.113599

Peer reviewed

HHS Public Access

Author manuscript

J Immunol Methods. Author manuscript; available in PMC 2024 March 21.

Published in final edited form as:

J Immunol Methods. 2024 February ; 525: 113599. doi:10.1016/j.jim.2023.113599.

Application of Enzyme-Linked Immunosorbent Assay to Detect Antimicrobial Peptides in Human Intestinal Lumen

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Abstract

Intestinal transplantation is the definitive treatment for intestinal failure. However, tissue rejection and graft-versus-host disease are relatively common complications, necessitating aggressive immunosuppression that can itself pose further complications. Tracking intraluminal markers in ileal effluent from standard ileostomies may present a noninvasive and sensitive way to detect developing pathology within the intestinal graft. This would be an improvement compared to current assessments, which are limited by poor sensitivity and specificity, contributing to under or over-immunosuppression, respectively, and by the need for invasive biopsies. Herein, we report an approach to reproducibly analyze ileal fluid obtained through stoma sampling for antimicrobial peptide/protein concentrations, reasoning that these molecules may provide an assessment of intestinal homeostasis and levels of intestinal inflammation over time. Concentrations of lysozyme (LYZ), myeloperoxidase (MPO), calprotectin (S100A8/A9) and β-defensin 2 (DEFB2) were assessed using adaptations of commercially available enzyme-linked immunosorbent assays (ELISAs). The concentration of α-defensin 5 (DEFA5) was assessed using a newly developed sandwich ELISA. Our data support that with proper preparation of ileal effluent specimens, precise and replicable determination of antimicrobial peptide/protein concentrations can be achieved for each of these target molecules via ELISA. This approach may prove to be reliable as a clinically useful assessment of intestinal homeostasis over time for patients with ileostomies.

Keywords

Intestinal transplantation; Transplantation immunology; ELISA; Methods; Crohn's disease

Introduction

Intestinal transplantation (ITx) is the definitive treatment for intestinal failure.[1,2] However, ITx intervention is limited by high rates of rejection and graft-versus-host disease (GVHD) compared to that of other solid organ transplants.[3 4,5] These complications necessitate aggressive immunosuppression, which carry the risk of adverse events, such as posttransplant lymphoproliferative disorder and infections.[6–8] Despite the profound consequences of rejection, current methods of early diagnosis where intervention might be possible have shortcomings, including endoscopic biopsy, the current gold standard for diagnosing rejection. Patchy distribution of rejection may require multiple invasive biopsies for accurate histopathology,[9] and the subjective nature of biopsy interpretations, such as "borderline rejection", can result in indecisive management.[10] As rejection episodes can occur many times over the course of multiple years post-transplant, there is a need for reliable, resource-light methods for the diagnosis and treatment of rejection after ITx.

Antimicrobial peptides (AMPs) help mediate mucosal homeostasis in the intestine with luminal microbes. Changes in AMP levels within secretory granules of intestinal Paneth cells have been associated with intestinal graft rejection as well as Crohn's disease.[11,12] We hypothesize that levels of AMPs in the ileal effluent might associate with rejection; if so, they could be serially monitored from stoma non-invasively and serve as biomarkers for early ITx rejection, which otherwise requires histological analysis of biopsy specimens.[13] To date, measurement of AMPs in ileal fluid through stoma sampling has not been reported. Of interest would be analysis of myeloperoxidase, calprotectin, β-defensin 2 (HBD2, DEFB2) and lysozyme; ELISA kits for these targets are commercially available but have not been adapted for use in ileal effluent. Another attractive target is α-defensin 5 (HD5, DEFA5), an abundant Paneth cell-specific effector.[14] Commercial ELISA kits are not available for HD5. Creating an HD5-specific ELISA would permit correlation of prior findings of mucosal HD5 to luminal HD5. Together, assessment of levels in ileal fluid through stoma sampling of these five AMPS might provide a noninvasive method of assessing mucosal innate immune barrier function, Paneth cell function, and indirectly, dysbiosis. In the present study, we describe the development and optimization of assays to detect AMPs in ileal stomal effluent, including the development of a sandwich ELISA assay for HD5.

Materials and methods

Materials

The commercial ELISA kits included lysozyme (Immunodiagnostik, Germany, REF KR6900), calprotectin (Immunodiagnostik, Germany, REF KR6927), myeloperoxidase (MPO) (Immunodiagnostik, Germany, REF KR6630), and β-defensin 2 (HBD2) (Immunodiagnostik, Germany, REF KR6500). Polyclonal anti-rabbit goat antibody conjugated to horseradish peroxidase (HRP) served as a secondary detection antibody (BD

Biosciences, New Jersey, USA, 554021). High-bind polystyrene 96-well plates (Corning, North Carolina, USA, REF 9018), phosphate buffered solution (PBS) (Corning, North Carolina, USA, 21–040-CV) and 20x concentrated phosphate buffered solution with 0.05% Tween (PSBT) (Thermo Scientific Pierce, Illinois, United States, REF 28352). Bradford assay was used to estimate total protein to normalize protein concentrations (Thermo Fisher Scientific, Massachusetts, USA, REF 23200). 3,3',5,5'-tetramethylbenzidine (Sigma Aldrich, Massachusetts, USA, REF T4444) was used as substrate at 1x dilution, as recommended by the manufacturer. Bradford assays were utilized rather than bicinchoninic assays (BCA), as prior studies have suggested that bilirubin can chelate with copper,[15] a key component of BCA.

Ileal Effluent Specimens

All ileal effluent samples were collected from a consecutive series of adult patients from a single institution under an institutional review board (IRB) approved protocol (protocol numbers AAAT7647 and AAAU1954). Negative control samples were collected from resected ileum from deceased donors and patients undergoing oncologic right colectomy. Positive control samples were collected from resected ileum from Crohn's disease patients undergoing right colectomy.

Defensin Peptides

HD5 and HD6 (DEFA6) were synthesized, folded, verified, and lyophilized as described previously.[16,17] For sandwich ELISA, serial dilutions of HD5 peptide were made in phosphate buffered saline (PBS) containing bovine serum albumin (Sigma-Aldrich) as carrier protein at a concentration of 0.1 g/ 100 ml.

Defensin Antibody Generation

For immunizations, synthetically prepared HD5 and HD6 peptides were chemically coupled to ovalbumin using glutaraldehyde as described.[18] Briefly, HD5 peptide (0.79 mg) and chicken ovalbumin (1.8 mg, Sigma A-2512) were dissolved in 1.0 ml PBS (yielding a molar ratio of ~10:1, peptide:ovalbumin). An equal volume of 0.2% (v/v) glutaraldehyde in PBS (diluted from 50% aqueous stock solution, Sigma G7651) was added, and the resulting solution was stirred at room temperature for 1 hr. The reaction was quenched by the addition of 0.5 ml of 1M glycine (Thermo Fisher Scientific, Waltham, MA). The quenched reaction solution was then transferred to a SpectraPor membrane pouch (molecular weight cut-off 3500 kDa; Spectrum Laboratories, Inc., New Brunswick, NJ) and dialyzed overnight against 1 liter of PBS at 4° C. The resulting conjugated HD5-ovalbumen preparation was then aliquoted (800μg/ml) and stored at −20° C until use as an antigen. The HD6-ovalbumen conjugated antigen was identically prepared.

For polyclonal antiserum generation, New Zealand Black rabbits were immunized by Antibodies Inc. (Davis, CA) with the HD5 peptide/OVA conjugate (200 μg) in Freund's complete adjuvant on day 1, and then boosted with the antigen in incomplete Freund's adjuvant on days 14, 21, 35 and 49. Preimmunization sera was collected as control antisera. Animals were humanely euthanized on day 57 and immune serum was harvested. All procedures were in accordance with relevant guidelines and regulations and approved by

the U.S. Department of Health and Human Services Public Health Service Animal Welfare Assurance Committee (Assurance ID D16–00576).

For monoclonal antibody production, mice were immunized by Antibodies Inc. (Davis, CA) with the HD5-peptide/OVA conjugated antigen, or the HD6-peptide/OVA conjugated antigen using an immunization protocol as described.[19,20] Serum from the immunized mice was analyzed to confirm positive immunoreactivity using a direct enzyme-linked immunosorbent assay (ELISA) for HD5 or HD6 target ligands, respectively.[20] After confirmation of positive immunoreactivity, mice were humanely euthanized; splenocytes were harvested at necropsy and then fused to create hybridomas.[20] All procedures were in accordance with relevant guidelines and regulations and approved by the U.S. Department of Health and Human Services Public Health Service Animal Welfare Assurance Committee (Assurance ID D16–00576).

For HD5 monoclonal antibodies, nascent hybridomas were plated in 96-well microtiter plates for cell culture. After incubation for one week, the cell culture supernatants were used in direct HD5 ELISA screens using plates coated with synthetic HD5 as target ligand and a balanced cocktail of IgG1, IgG2a and IgG2b subclass-specific anti-mouse secondary antibodies for detection.[20] Approximately 100 candidate hybridoma cultures from the 96-well plates were identified, selected, and expanded in 24-well culture plates. After further screening by ELISA, four top candidate clones (three IgG1 and one IgG2a) were then subcloned to homogeneity by limiting dilution.[20] Hybridoma clone CB/HD5/65.1.3 was strongly positive for HD5, was determined to be isotype IgG2a and had no immunoreactivity by ELISA against ovalbumin or human defensin 6 (HD6) as negative controls. This clone was expanded by growth in a Corning CELLine Disposable Bioreactor (Antibodies Inc.) using a protocol based on the manufacturer's guide. The bioreactor supernatant (0.9 mg/ml protein) was used for subsequent ELISA and immunofluorescence experiments.

Histochemistry and Fluorescence Immunohistology

Human jejunum was fixed in 4% w/v paraformaldehyde, paraffin embedded, and then sectioned (4–5 μM) and mounted on X-tra™ positive-charged slides (Leica Biosystems, Buffalo Grove, IL) as described.[21,22] Mounted tissue sections were deparaffinized, then rehydrated in gradient ethanol and H_2O. For histology and light microscopy, tissue was stained with Masson's trichrome. For immunofluorescence histology, antigen retrieval was performed on hydrated tissues by incubating slides overnight in a water bath at 65ºC in Coplin jars containing Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 9.0). Following antigen retrieval, tissue sections were rinsed with PBS and blocked with 5% donkey serum (in PBS) for 30 min prior to overnight incubation at 4°C with primary antibodies: anti-HD5 rabbit polyclonal serum (reported here, 1:750), mouse monoclonal antibody CB/HD5/65.1.3 (reported here, 1:1000), mouse monoclonal CB/HD6/1.1(reported here, 1:1000), and anti-human lysozyme rabbit polyclonal serum (A0099, Dako/Agilent, Santa Clara, CA, 1:4500). Following overnight incubation with primary antibodies, tissue sections were rinsed with PBS and then incubated with secondary antibodies: goat anti-mouse IgG Alexa Fluor Plus 647 (ThermoFisher Scientific) and goat anti-rabbit IgG Alexa Flour 488 (Abcam, Cambridge MA), rinsed, and then stained with DAPI using the TrueVIEW

Autofluorescence Quenching Kit (Vector Laboratories, Burlingame, CA) as outlined by the manufacturer. Confocal images were acquired using a Leica SP8 STED 3 × microscope (Leica Microsystems Inc., Buffalo Grove, IL). Confocal Z-stack images were generated with Fiji ImageJ (version 2.9.0, https://imagej.net/Fiji) software.[23]

Modification of commercial ELISA kit protocols

Commercial ELISA kits originally developed for stool were adapted for analysis of ileal effluent. For lysozyme, calprotectin, and HBD2, ileal effluent samples were clarified by centrifugation for 20 minutes at 14,000 relative centrifugal force (RCF) at 4°C to remove large particulate matter. Because of manufacturer recommendations to minimize mechanical disruptions of test samples, this step was excluded for myeloperoxidase. In the first iteration, samples were diluted to manufacturer recommendations, which had been validated in stool. Subsequent iterations were conducted using altered sample dilutions to capture measurements for most ileal effluent samples.

HD5 Sandwich ELISA Development and Optimization

Upon collection, ileal effluent samples were aliquoted into 0.5 to 1 mL samples and stored in a liquid nitrogen freezer. Individual aliquots were removed and thawed on ice prior to use by ELISA method. Samples were diluted in phosphate-buffered saline including 0.05% tween (PBST). Diluted samples were centrifuged for 20 minutes at 14,000 RCF and 4°C and filtered through a 40 μm filter for homogenization. Previously developed sandwich ELISAs, optimized for urine,[24] served as a guide in developing the reported ELISAs herein. High-bind polystyrene plates were coated with 1 μg/mL mouse monoclonal anti-HD5 antibody in PBS (1 μg/mL in PBS, 100 μL/well). Plates were incubated overnight at 4°C, then washed with PBS, and then blocked overnight at 4°C with 1% bovine serum albumin solution in PBST (300 μL/well). Plates were then washed with PBS prior to the ELISA. Standard curves used synthetic HD5 at concentrations ranging from 1,000 to 100,000 pg/mL (100 μL/well). Ileal effluent samples were prepared at dilutions between 1:50 and 1:3200 (100 μL/well). Four negative controls, each missing one of the four components (capture antibody, substrate, primary antibody, and secondary antibody) were plated in duplicate to rule out nonspecific binding. Standards, ileal effluents, and controls were incubated for one hour at 37°C. Plates were washed with PBST, then plated with 1:1000 polyclonal rabbit antibody as primary detection antibody (100 μL/well) at room temperature for one hour. Plates were washed with PBST, then plated with 1:1000 goat anti-rabbit polyclonal antibody (100 μL/well) as secondary detection antibody, and incubated for one hour at 37°C. After washing with PBST, TMB (1x solution, 100 μL/well) was added as a substrate for HRP. The plate was incubated at room temperature in the dark until differentiation was identified between the standards. The reaction was quenched with 1M H_2SO_4 (100 μL/well). Absorbance at 450nm and 620nm (Abs_{450} and Abs_{620}), representing peak absorbance of processed substrate and background, respectively, were determined using Biotek Synergy H1 Microplate Reader. $Abs_{Blank450}$ and $Abs_{Blank620}$ were calculated at 450nm and 620nm, respectively, as the difference between absorbance of wells containing sample and the average absorbance of 12 blank wells. $\Delta Abs_{450–620nm}$ was then calculated as the difference between $Abs_{Blank450}$ and $Abs_{Blank620}$. A 4-parameter curve was calculated from the standards' $\Delta Abs_{450–620nm}$ and known concentrations using the

calculations provided by the spectrophotometer software, Gen5 v3.02, or Graphpad Prism v9.3.0. Sample concentrations were approximated based on their $\Delta Abs_{450–620nm}$ and the 4-parameter curve.

HD5 Sandwich ELISA Validation

Additional steps were taken to validate the newly developed HD5 ELISA. Limit of blank, limit of detection, and limit of quantification were determined as described by Armbruster et al.[25] Briefly, limit of blank was estimated using wells containing only the diluent (PBST) using the following equation: Limit of blank = Average $concentration_{Blanks}$ + 1.645*($stdev_{Blanks}$). The lower limit of dilution was estimated using the limit of blank and the standard deviations of representative low concentration samples, which was defined as samples with less than 100 pg/mL. As multiple samples qualified as a low concentration sample, the standard deviation used in the equation was the average of all standard deviations across low concentration samples: Lower limit of dilution = Limit of blank + 1.645*($stdev_{Low\ concentration\ sample}$), where $Stdev_{Low\ concentration\ sample}$ = average(Σ(stdev)). A lower limit of quantification was defined as the higher of two values between the lower limit of dilution and lowest level measured, given that intra-assay coefficient of variation was acceptable at that measurement (CV% below 10%). Intra-assay CV% was calculated from duplicate samples from a single plate: Intra-assay coefficient of variation (CV%) = (standard deviation)/(average)*100%. The linearity of dilution was represented as a plotted line between the observed concentration and the expected concentration for each dilution. Expected concentrations for each dilution were calculated from the concentration of the previous dilution and the dilution fold: Expected $concentration_b$ = Observed $concentration_a$ * ($dilution\ fold_{Between\ a\ and\ b}$).

Percent coefficient of variation (CV%)

Each commercial ELISA was conducted twice using the same five samples (S1-S5). Four samples (S3, S10-S12) were replicated for the HD5 ELISA. Percent coefficients of variation (CV%) were calculated between replicates in a single assay (intra-assay) and replicates across plates (inter-assay). Standard deviations of replicates were divided by average of replicates and multiplied by 100%.

Spike and recovery analysis

A spike and recovery assay was performed for four different ileal effluent samples using the HD5 ELISA. Ileal effluent samples were diluted in PBST. 228 μL of each diluted sample were isolated and combined with 12 μL of highly concentrated HD5 standard (500,000 pg/mL). This resulted in a spike of 6,000 pg in 240 μL, resulting in an expected increase in concentration of 25,000 pg/mL at each dilution.

Results

Generation and specificity validation of capture and detection HD5-antibodies

To establish a method to quantitate HD5 in intestinal luminal effluent, we sought to develop a sandwich ELISA using a mouse monoclonal HD5-directed immunoglobulin as a capture antibody, and a rabbit polyclonal immune serum as an indicator. Previous experience in

the field demonstrated that useful antibodies for α-defensins were challenging to generate. Specifically, the small size and highly folded nature of defensins, created by intramolecular tri-disulfide bonds, necessitates properly folded α-defensin peptides to generate optimal antigens.[26,27] Accordingly, HD5 was chemically synthesized, folded, and verified for purity and proper folding as described.[16,17] Using this synthetic peptide as antigen, multiple hybridoma cell lines secreting mAbs against HD5 were isolated following mouse immunization. Following multiple rounds of screening and subcloning by limiting dilution to homogeneity,[20] hybridoma clone CB/HD5/65.1.3 was strongly positive for HD5 binding, was determined to be isotype IgG2a and had no immunoreactivity by ELISA against either ovalbumin (the carrier protein used for immunization) or HD6 as negative controls. A representative immunohistochemistry image using CB/HD5/65.1.3 shows specific staining and colocalization of signal with lysozyme in Paneth cell granules Figure 1B. Similarly, the rabbit polyclonal antiserum generated to synthetic HD5 shows specific staining and colocalization of signal with HD6 in Paneth cell granules (Figure 1C).

Bradford total protein assay - Replicability

Bradford total protein assay was found to have acceptable replicability. Intra-assay CV% for five repeated and five non-repeated samples ranged between 0.07 to 8.8%. Inter-assay CV% for five replicated samples ranged from 0.7% to 27.2%.

Commercial ELISAs for calprotectin, HBD2, MPO, and lysozyme - Replicability

Intra and inter-assay CV% for five samples analyzed in duplicate wells in two different plates are shown in Supplemental Table 1a–d. Of the ten intra-assay measurements determined for each AMP, few assay measurements exceeded the acceptable 10% boundary (calprotectin [3 out of 10], HBD2 [0/10], MPO [2/10], lysozyme [1/10]). Of the five inter-assay measurements, several exceeded the 15% boundary (calprotectin [1/5], HBD2 [4/5], MPO [3/5], lysozyme [1/5]) when associated with lower concentrations. Of these, only the three MPO ELISA anomalous measurements exceeded a 40% coefficient of variation.

Commercial ELISAs - Linearity of dilution

Figure 2 depicts the relationship between observed concentration of five samples at various dilutions and the expected concentrations at each dilution. All four AMP ELISAs displayed excellent linearity of dilution (Figure 2).

HD5 ELISA results – Replicability and linearity of dilution

Percent coefficient of variation (CV%) was calculated for assays of the HD5 standard between assays of wells within a single plate and those across different plates (Supplementary Table 2). All intra-plate assay CV%’s were within an acceptable range below 10%. Six of eight HD5 standards had inter-assay CV%’s that were below 15%. One of the eight standards was slightly above acceptable levels at 16.6%, and another had an inter-assay CV% of 29.8%. The working range of this assay as determined by the range where CV% between replicates was <10% was from 1,000 pg/mL to 25,000 pg/mL. Four repeated standard curves as a function of HD5 concentration and ELISA absorbance are shown in Figure 3.

Linearity of dilution was confirmed for four samples tested by HD5 ELISA (Figure 4).—CV% values were calculated for two samples (with six dilutions between 1:10 and 1:31250) that were run in triplicate across two plates. Intra-assay CV% ranged from 1.44% to 7.99% for all but one sample, which was 10.50%. Inter-assay CV% across two plates ranged from 5.71% to 14.91% for five of six replicates, while it was 26.21% at the lowest dilution (Supplementary Table 3). HD5 measurements across repeated samples are shown in Figure 5.

Limit of blanks, limit of detection, and limit of quantification for HD5 ELISA

A limit of blanks was calculated to be 0.0 from 24 blank wells. A limit of detection was estimated to be 230 pg/mL, based on a calculation from the limit of blanks as well as samples with values up to 10,000 pg/mL. A lower limit of quantification was calculated as the higher value between limit of detection and the lowest value with intra-assay CV% below 10%. This value was approximately 500 pg/mL.

HD5 results - Spike and recovery

Adding a constant spike of 25,000 pg/mL HD5 to three samples at nine different dilutions between 1:50 and 1:6400 resulted in varying recovery. Spiked samples at dilutions of 1:400 or less had percent recovery ranging between −3% to 73%. At dilutions at 1:800 or above, percent recovery ranged from 83% to 103.6% for S11, 83% to 106% for S12, and 66% to 78% for S13.

Discussion

A noninvasive technique for measuring AMP levels in luminal fluid following intestinal transplantation may provide a novel method of diagnosing rejection and inform understanding of the mechanism of rejection. In particular, quantifying AMPs in the intestinal lumen may reflect a possible role of an imbalance between mucosal defenses and the gut microbiome in intestinal transplant rejection.[28–32] Moreover, elucidating the pathophysiology of rejection in intestinal transplant rejection may expand our understanding of other pathologies, such as Crohn's disease.[33] Both pathologies exhibit changes in AMP production by Paneth cells,[22,34–36] and both may be related to *NOD2*,[37–39] a gene associated with decreased AMP production (HD5 and HBD2) by Paneth cells.[34,40,41]

Currently, there are no established protocols for measuring intraluminal levels of AMPs that are cost-effective and scalable. Proteomic analyses and metabolomic analyses achieved by advanced methods such as Luminex and mass spectrometry have been helpful in identifying differences in luminal content between patients with and without intestinal graft rejection.[42,43] However, such methods are costly and risk contaminating highly sensitive instruments.[44] Enzyme-linked immunosorbent assays (ELISAs) are relatively low-cost and high-throughput, are already utilized by clinical laboratories, and exist for detection of several AMPs in stool. However, a complication of monitoring stool samples is the variable AMP degradation that can occur over time in fecal material.[45,46] In contrast to stool, the collection of ileal effluent through ileostomies in most intestinal transplant recipients is

practical and straightforward,[47] offering kinetic parameters to avoid degradation and permit accurate AMP measurements to evaluate for intestinal graft rejection.[48,49]

Similar to stool, ileal effluent is a heterogeneous and complex matrix capable of significant variation in water, fat, protein, and indigestible fiber content.[50] In both matrices, heterogeneity can be improved by normalizing measured protein to total protein content, as has been done in stool ELISAs.[51,52] While a few studies have repurposed commercially available fecal ELISAs for ileal effluent, they did not provide information on validity or optimization for this complex matrix.[53,54]

Our data support that ELISAs are precise and replicable in ileal effluent following thorough homogenization. Accuracy and specificity for the commercial ELISAs were not tested in our lab. Accuracy and specificity were tested for the HD5 ELISA assay using spike and recovery and histopathology (Figure 1), respectively, though further confirmation can be pursued via mass spectrometry, western blots, or liquid chromatography. The new sandwich ELISA for HD5 proved to be a replicable assay. This assay uses newly developed capture and detection antibodies generated to an HD5 peptide that was chemically synthesized, folded, and verified in line with highest standards in the field.[16,17] Specificity of these reagents was demonstrated by immunofluorescence microscopy, where Paneth cell granules were specifically labelled (Figure 1). The HD5 ELISA appears to be robust against inter-assay variability. The newly developed HD5 ELISA showed higher variation at higher ligand concentrations. However, extending the range of dilutions of ileal effluent can allow for precise measurements, as demonstrated in Figure 5. In contrast, the commercial ELISAs for AMPs seemed to lose precision at lower concentrations. Variation within a single ELISA was within acceptable values (~10%). Instances where inter-assay variation exceeded this threshold coincided with low concentrations (Supplementary Table 1). However, low concentrations of any of the AMPs tested in our ELISAs may be lower than needed. For example, samples with low calprotectin levels (1.7 to 2.6 ug/mL) are lower than normal levels of fecal calprotectin (10–50 ug/mL),[55] and far lower than fecal calprotectin levels in active inflammatory bowel disease (>250 ug/mL).[56]

As for limitations, some of the intra and inter-assay CV% exceeded ideal values, suggesting that homogenization methods could be further improved. Additionally, experimental parameters, such as incubation times and temperatures, for the HD5 ELISA could be further refined. Furthermore, HD5 ELISA was not tested for specificity in this experimental set. Commercial ELISAs were not tested for sensitivity and specificity by our lab but previously have been tested by their respective manufacturer. As these tests were performed in other matrices (e.g., urine and stool), perhaps it would be beneficial to determine sensitivity and specificity in ileal effluent. Finally, MPO is a protein with tertiary and quaternary structure consisting of two light and two heavy chains. Concern for disrupting its complex structure limited the intensity of homogenization of ileal effluent samples and may have impacted results.

While all ELISAs were performed by a single investigator, replicates were performed on multiple days spanning multiple months under different laboratory conditions, including ambient temperature, lighting, and incubation times. We believe that if scaled up, these

ELISAs, used to assay ileal effluent, will be forgiving of small variations in protocol and can be reliably utilized across clinical laboratories with little to no additional training.

Conclusion

Our adapted and novel ELISAs achieve consistent values in serial measurements of ileal effluent samples. With attentive sample processing, existing and newly developed ELISAs can likely be optimized for future application in ileal effluent. Further efforts to confirm accuracy and specificity in ileal effluent will be helpful. Such ELISAs may prove to be a reliable and low-cost method that can be used easily and repeatedly to assess levels of intestinal inflammation and gut homeostasis over time. Moreover, this approach may be clinically applicable in the assessment of patients with ileostomies related to inflammatory bowel disease or intestinal transplantation. Future efforts are also needed to ascertain whether the measurements obtained with our assays correlate with clinical findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We acknowledge the expert technical assistance of Dr. Anchasa Kananurak. We thank Drs. Belvin Gong and James Trimmer, and the staff of the UC Davis/NIH NeuroMab Facility, for expert assistance in the development of monoclonal antibodies. We thank Dr. Ingrid Brust-Mascher at the Health Sciences District Advanced Imaging Facility (UC Davis) for assistance with confocal microscopy.

Funding

This work was supported by K23 GG016887, AI125926, and AI32738. An institutional T32 (AI148099–04) supported Julie Hong, and an institutional T35 (DK093430–10) supported Abrar Shamim for the duration of this project.

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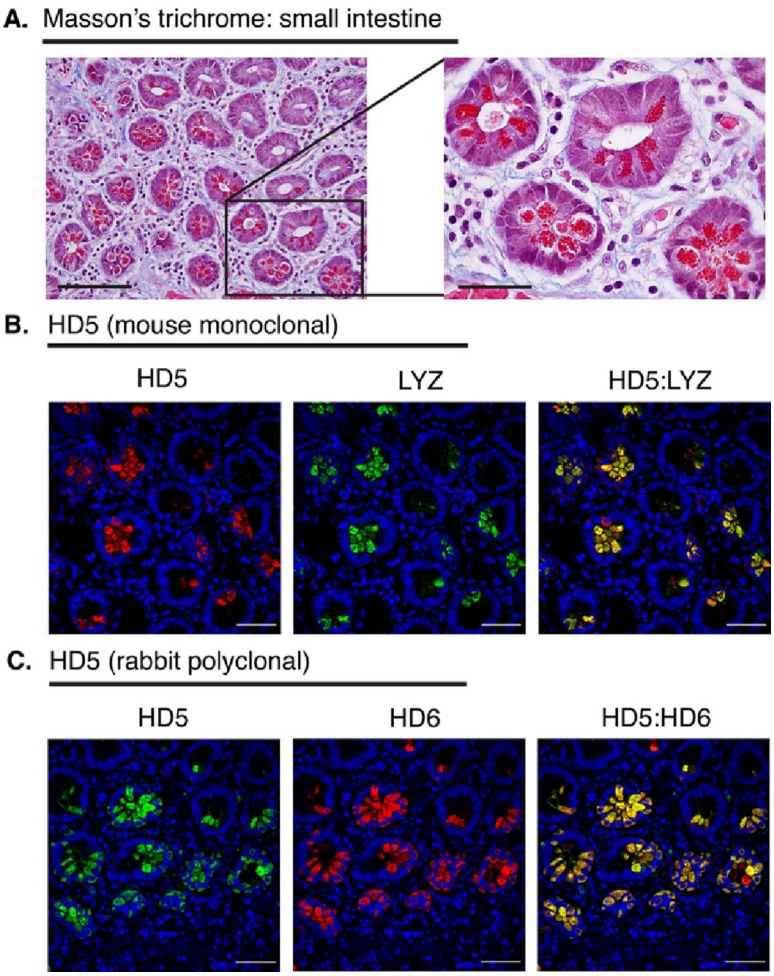

Figure 1. Human jejunum specimens analyzed by Masson's trichrome histochemistry (top panels) and fluorescence immunohistology (middle and bottom panels).

A. Masson's trichrome staining highlights Paneth cells at base of crypts with intensely eosinophilic granules (oriented as cross sections). B. Immunofluorescent signal of mouse HD5 monoclonal antibody (CB/HD5/65.1.3, red, left panel) and signal of rabbit anti-lysozyme polyclonal immunoserum (green, middle panel) colocalize in Paneth cells (yellow, right panel). C. Immunofluorescent signal of rabbit HD5 polyclonal immunoserum (green, left panel) and signal of mouse HD6 monoclonal antibody (CB/HD6/1.1, red, middle panel) colocalize in Paneth cells (yellow, right panel). Scale bars: light microscopy 100 μm (left) and 50 μm (right); fluorescence microscopy 50 μm.

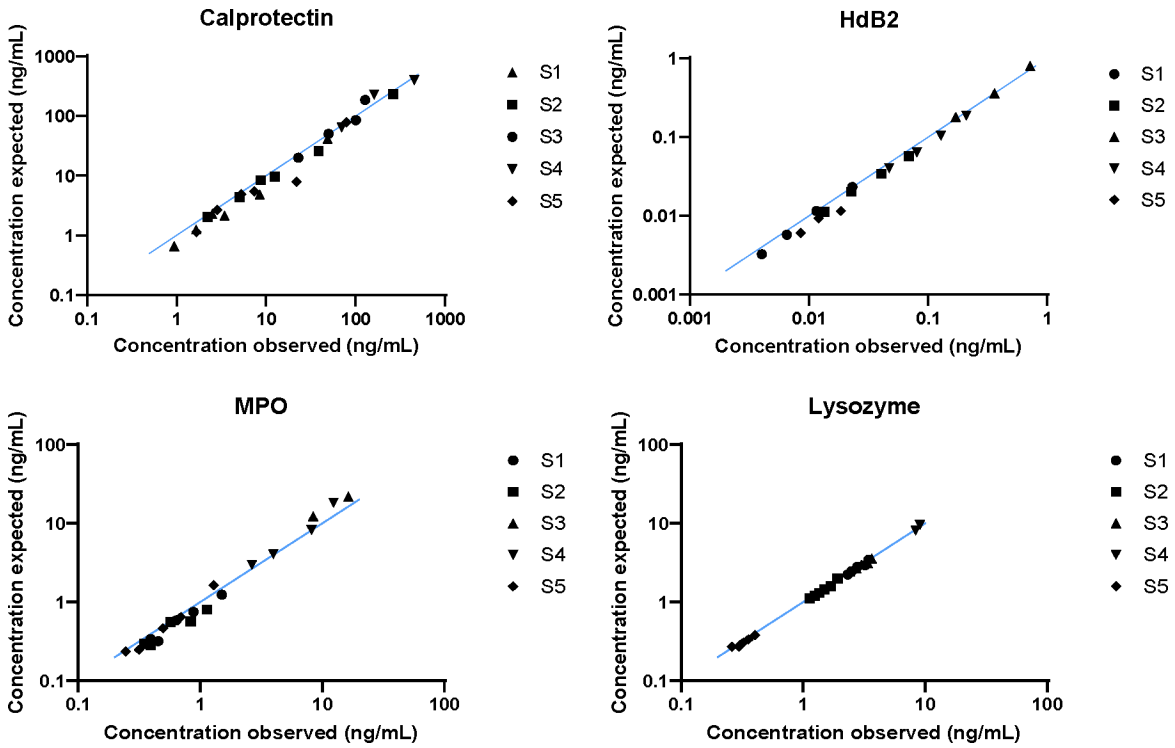

Figure 2. Linearity of dilutions of samples in commercial ELISAs.

Linearity of dilution for four antimicrobial peptides (calprotectin, HBD2, MPO, lysozyme) represented by graphing observed concentration of samples at different dilutions against calculated concentrations. Calculated concentrations were determined for each assay based on the concentration of the previous serial dilution. Blue trendline represents where linearly diluted samples would fall such that observed concentrations match expected concentrations.

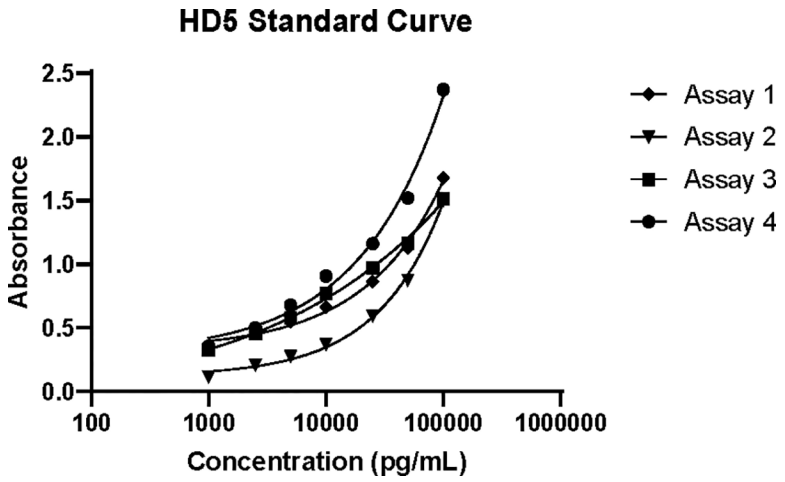

Figure 3. Replicability of HD5 standard curve.

One standard curve of HD5 for concentrations ranging from 1,000 to 100,000 pg/mL. Average delta absorbances (Abs) were calculated by subtracting spectrophotometer readings of 620nm from 450nm.

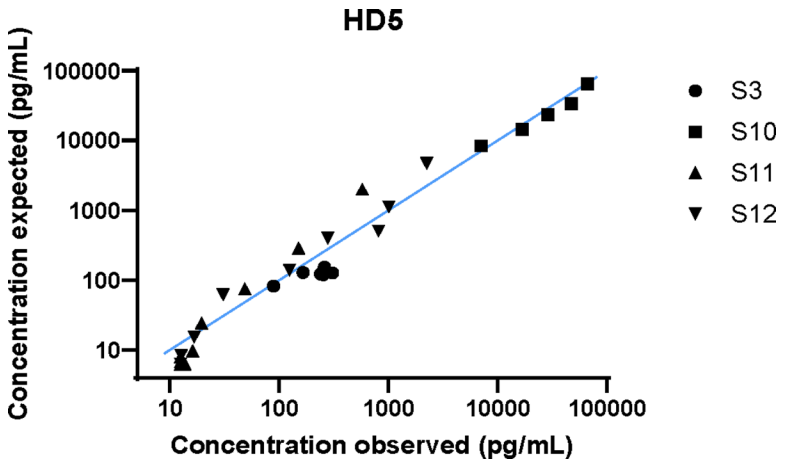

Figure 4. Linearity of dilution of samples of HD5.

Linearity of dilution represented by graphing observed concentration of samples at different dilutions against expected concentrations. Expected concentrations were calculated for all dilutions based on the concentration of the previous serial dilution. Blue trendline represents where linearly diluted samples would fall such that observed concentrations match expected concentrations.

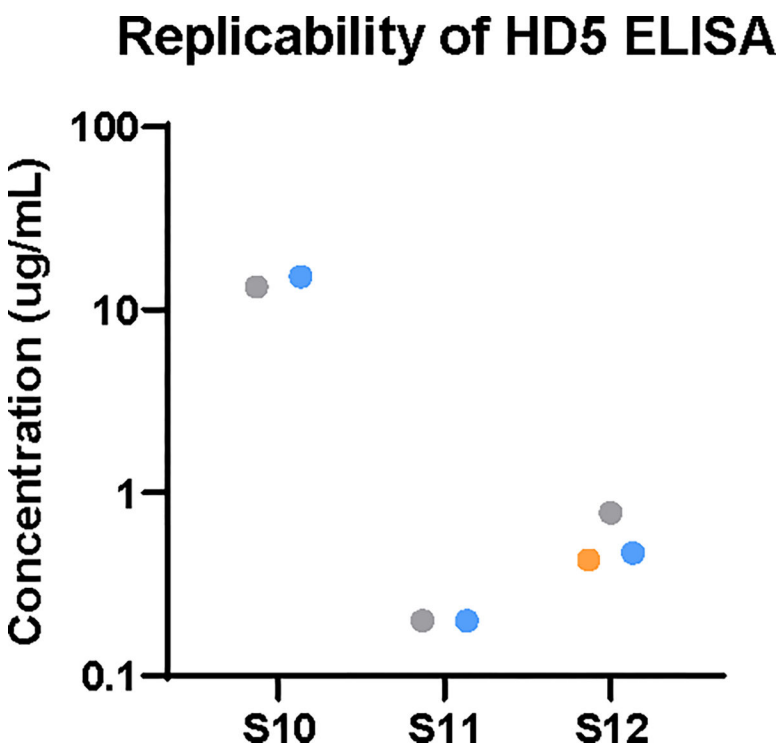

Figure 5. Replicability of measurements using HD5 ELISA.
Average concentration of samples repeated across multiple HD5 ELISA assays

Table 1.
Concentrations of AMPs normalized to Bradford protein assay.

Average concentration of four antimicrobial peptides (AMPs) normalized by total protein concentration as calculated by Bradford protein assay for five different samples of ileal effluent collected from different patients. All concentrations of normalized AMPs represented as ug/mg. Concentrations of total protein as estimated by Bradford assay are represented as mg/mL.

	Total protein (mg/mL)	Calprotectin (μg/mg)	HBD2 (μg/mg)	MPO (μg/mg)	Lysozyme (μg/mg)
S1	14.44	0.13	0.10	0.02	0.04
S2	10.08	1.39	0.16	0.03	0.03
S3	17.92	17.03	18.72	1.73	0.04
S4	54.38	18.43	0.20	0.22	0.04
S5	3.54	0.88	0.59	0.20	0.02

Abbrev: HBD2 (*β*-defensin 2), MPO (myeloperoxidase), S1-5 (samples 1-5).