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A Multiplexed Assay to Detect Antimicrobial Peptides in Biological Fluids and Cell Secretions

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Abstract

Mucosal tissues represent the front line in defense against potential pathogens, and one means by which mucosa provide protection is via the secretion of antimicrobials which can interfere with potential pathogens as well as recruit and modify the responses of immune cells. Here we describe adaptation of ELISA assays to microsphere format, facilitating simultaneous quantification of antimicrobial peptides including elafin, MIP3, HBD2, HBD3, SLPI, RANTES, SDF1, lactoferrin, LL-37, and HNP1-3. The multiplexed assay exhibits excellent reproducibility, shows linearity over a two order of magnitude concentration range for most analytes, is compatible with biological fluids such as cervicovaginal lavage fluid, and presents significant cost and sample savings relative to traditional ELISA assays.

Keywords

Antimicrobials; female reproductive tract; mucosal secretions; chemokines; cytokines; multiplex assay; defensins

1. Introduction

Mucosal tissues are susceptible portals of entry for many human pathogens that are a major cause of infectious disease worldwide (Woodrow et al., 2012). The female reproductive tract (FRT) alone is vulnerable to infection from over 20 sexually transmitted pathogens including *N. gonorrhoeae*, *C. trachomatis*, HSV and HIV among others (Wira et al., 2011). Development of prevention and treatment methods for sexually transmitted infections remains a challenging area of research.

The mucosal epithelium of the gastrointestinal, upper respiratory, nasopharyngeal, mammary, lacrimal, and FRT tracts can actively combat infection by pathogens by secreting

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antimicrobial peptides as well as cytokines and chemokines that act as anti-microbial agents (reviewed in McGhee and Fujihashi, 2012). Collectively, these antimicrobial factors have a wide range of effects; RANTES, SDF1, HNP1-3, HBD2-3, SLPI and elafin are effective against HIV-1, the causative agent of AIDS, as well as bacteria, fungi, and other enveloped viruses (Schneider et al., 2005; Wiesner and Vilcinskas, 2010). Importantly, antimicrobial peptides are often multifunctional, performing anti-pathogen as well as chemotactic and other necessary homeostatic functions such as recruiting effector cells directly or indirectly by stimulating chemokine or cytokine production (Yang et al., 2004). Additionally, secreted AMPs can perform their functions while sparing essential commensal flora, such as *L. crispatus* (Wira et al., 2011).

The detection and quantification of antimicrobial factors in biological fluids is important for identification and characterization of a variety of anti-pathogenic effects, and can be used to elucidate the cellular origin of these factors. The most common method of analyzing antimicrobial in secretions from the FRT and other mucosal tissues is by ELISA (Fahey et al., 2005). Although this method performs reasonably well, it requires a large sample size, can measure only one factor at a time, and is relatively costly. Further, despite the availability of multiplex assays for some cytokines and chemokines, to the best of our knowledge, no platform has been developed that is dedicated to measuring antimicrobials in biological fluids and cell secretions.

We report the development of a customized multiplex microsphere assay that permits simultaneous detection of multiple antimicrobials from FRT-derived secretions that are known to inhibit HIV. Our method performs comparably to or better than ELISA, is multi-factorial, economical, and most significantly, has greatly reduced sample volume requirements. While we show the advantages of a multiplex assay for measuring antimicrobial agents found in the FRT, this method could easily be applied to identifying antimicrobial agents found in a variety of biological fluids including saliva, stool, and in the mucosal linings of the respiratory and intestinal tracts.

2. Materials and Methods

2.1 Antibodies and standard curve analytes

Capture and detection antibodies, as well as antimicrobial factor standards were sourced as described in Table 1. In some cases, special requests were made to manufacturers to supply the antibodies in the absence of carrier protein, typically bovine serum albumin (BSA), in order to facilitate microsphere conjugation.

2.2 ELISA assays

High binding polystyrene 96 well plates (Corning) were incubated with 100 μ l of 5 μ g/ml of capture antibody in phosphate buffered saline (PBS) overnight at 4°C. The plates were washed three times with 200 μ l of PBS 0.05% Tween-20 (PBS-T) and blocked with 100 μ l of PBS 1% BSA for 1 hr at room temperature. The plates were washed three times with 200 μ l of PBS-T and were incubated with analyte at the manufacturer's recommended concentrations and buffer conditions for 2 hr at room temperature. The plates were washed three times and incubated with detection antibody at the recommended concentration and buffer condition for 1 hr at room temperature. After detection, 100 μ l of Strep-HRP diluted 1:200 into PBS (R&D Systems) was incubated for 30 min at room temperature. The plates were washed three times with 200 μ l of PBS-T and 150 μ l of ABTS one-step substrate (Thermo Scientific) was added and incubated for 30 min at room temperature. The absorbance at 405 nm was measured using a UV/Vis spectrophotometer (Molecular Devices) at 25°C.

2.3 Preparation of capture antibody-conjugated microspheres

A customized multivariate microsphere assay was developed using a panel of capture antibodies coupled to carboxylated magnetic fluorescent microspheres (MagPlex-C Microspheres, Luminex Corp.) in an adaptation of a previously described method (Brown et al., 2012). A total of 1 million carboxylated microspheres were covalently coupled to 5 µg capture antibody using a two-step carbodiimide reaction. The antibodies used are listed in Table 1. Microspheres were washed by centrifugation and magnetic separation, then activated by resuspension in 80 µl of 100 mM monobasic sodium phosphate, pH 6.2, followed by the addition of 10 µl of 50 mg/ml N-hydroxysulfosuccinimide (24520, Pierce) in deionized water and 10 µl of 50 mg/ml 1-ethyl-3-[3 dimethylaminopropyl]carbodiimide-HCl (77149, Pierce) in deionized water. This reaction mixture was mixed end-over-end on an inverter for 20 min at room temperature. Activated microspheres were then washed three times in 150 µl of phosphate buffered saline (PBS), resuspended in 100 µl of PBS, and incubated with 5 µg capture antibody, in a final volume of 500 µl of PBS, on an inverter for 2 hrs at room temperature. Finally, coupled microspheres were washed with 500 µl of PBS and resuspended in 250 µl of PBS-TBN (PBS, 0.1% BSA, 0.02% Tween 20, 0.05% Sodium Azide, pH 7.4). After either 30 min at room temperature or an overnight incubation at 4°C in PBS-TBN, microspheres were washed with 500 µl PBS to remove blocking buffer and resuspended in 150 µl of PBSTBN. The coupled microspheres were counted on an automated cell counter (TC10, Biorad) and stored at 4°C in the dark for up to 12 months.

2.4 Preparation of standard curves and buffer selection

Recombinant human or human-derived antimicrobial peptides were sourced commercially as described in Table 1. Standard curves were generated using the manufacturer's recommended concentration ranges for the standard whenever it had been shown to include the dynamic range of fluorescence detection on the Luminex FlexMap-3D. For those where noticeable saturation could be observed at high concentrations, the maximum concentration was decreased. Standard curves were serially diluted 2-fold in PBS-T.

Several of the ELISA assays adapted to the microsphere format utilized proprietary buffers, necessitating buffer compatibility experiments. Therefore, standard curves using PBS 0.1% BSA (PBS-B) as dilution and wash buffer were compared to those generated using combinations of the HNP1-3 (Hycult Biotech) and LL-37 (Hycult Biotech) ELISA-kit buffers with PBS, PBS-T, and PBS-B and 10 mM acetic acid 10 mM sodium phosphate pH 7.2 at different steps in the protocol.

2.5 Fluorescent signal detection

A master mix of antibody-coupled microspheres was prepared by combining individual bead sets to achieve a final count of 500 microspheres of each specificity in a 10 µl final volume in PBS-T. Black, clear bottom 384-well plates (Greiner Bio One, 781906) were used. 40 µl of sample at 1.25x the target concentration was first added to the wells. Then 10 µl of the working microsphere master mix (500 microspheres of each type/well) was added. The plate was covered with microplate adhesive film (USA Scientific, 2920-0000), and the bottom of the plate was submerged in a sonicator for 15 seconds and then incubated on an XYZ-plane plate shaker (IKA, MTS 2/4) at room temperature for 2 hrs. After incubation, the plate was washed five times with 60 µl of PBS-T using a plate washer (Biotek, 405 Select TS). Next, biotinylated detection antibodies were mixed together at the vendor recommended concentrations and diluted in HNP1-3 ELISA kit dilution buffer, LL-37 ELISA kit dilution buffers (Hycult Biotech), PBS, PBS-T, PBS-B or 10 mM acetic acid 10 mM sodium phosphate pH 7.2. The plate was covered, sonicated and incubated for 1 hr at room temperature on XYZ plane plate shaker. Microspheres were washed five times with PBS-T as before. Then, Strep-PE (Prozyme, PJ31S) was added as a secondary detection reagent.

Strep-PE was diluted at 0.5 μ l /1000 μ l in HNP1-3 ELISA kit dilution buffer (Hycult Biotech) and 40 μ l of this solution was added to each well. The plate was covered, sonicated and placed on the XYZ shaker for 15 min. Five washes using 60 μ l of PBST were performed as before, and after the washes, the microspheres were resuspended in 35 μ l of Luminex Sheath Fluid. The plate was then covered, sonicated and placed in a Bio-plex array reader (FlexMap 3D, Bio-Plex Manager 5.0, Bio-Rad). The Median Fluorescence Intensity (MFI) of PE signal was determined for each bead set in each well. Background signal, defined as the average MFI observed for each microsphere set when incubated with detection reagent(s) in the absence of analyte, was subtracted from the MFI of each sample.

2.6 Preparation of clinical and ex vivo tissue culture samples

Cervical lavages (CVL) were obtained from participants in the HIV Epidemiology Research (HER) study at Miriam Hospital, Brown University, Providence, RI. All subjects gave written informed consent and studies were approved by the appropriate Institutional Review Board(s). CVL samples were collected by gently washing the cervicovaginal area with 10 ml of sterile normal saline (pH ~7.2). Following CVL collection, samples were immediately frozen at -80°C .

FRT tissues were obtained from women undergoing hysterectomies at Dartmouth-Hitchcock Medical Center (Lebanon, NH), who gave written informed consent before surgery. Studies were performed with the Dartmouth College Institutional Review Board approval and approval from the Committee for the Protection of Human Subjects (CPHS). Tissues obtained from hysterectomies included endometrium (EM), endocervix (CX) and ectocervix (ECX) and were transferred to the laboratory immediately after surgery. Tissues were rinsed with Hank's balanced salt solution (HBSS), minced under sterile conditions into 1-2 mm fragments and digested using a non-proteolytic enzyme mixture containing 0.05% collagenase type IV (Sigma-Aldrich, St. Louis, MO) and 0.01% DNase (Worthington Biochemical) for 1h at 37°C . After digestion, cells were washed and placed in culture for 48hr in Xvivo15 media clear (Lonza) supplemented with 10% stripped human AB serum (Valley Biomedical). The samples tested correspond to cell supernatants collected after 48hr of ex vivo cell culture.

CVL and secretions from cells grown in culture were serially diluted in 2-fold steps using HNP ELISA dilution buffer (Hycult Biotech). Prior to dilution, the samples were stored at -80°C and thawed at room temperature. In parallel, samples were also spiked with an equal volume of analyte standard mastermix at concentrations 2-fold higher than the midpoint of the standard curve, in order to determine whether all analytes, whether present natively in the particular samples tested or not, could be detected. Individual samples and standard curves were measured in duplicate.

2.7 Statistical analysis

Statistical analysis was performed in Prism (GraphPad), to determine Pearson correlation coefficients (PCCs) and coefficients of variation (CVs). A Wilcoxon matched pairs signed rank test was used to compare the CVs of matched microsphere and ELISA assays.

3. Results and discussion

3.1 Comparability with ELISA

ELISA and microsphere assays for 10 different antimicrobial factors were run using the same antibody pairs, buffers, and standard curves as recommended by the manufacturers. Dose-response curves for both ELISA and microsphere assays are presented in Figure 1A, and demonstrate excellent linearity over a 2 order-of-magnitude (OOM) range in analyte

concentration. In general, both methods provided comparable data; however, relative to the ELISA, greater sensitivity was observed for several microsphere assays (MIP3, HBD2, and SLPI). Thus, for all antimicrobial peptides tested, ELISA assays could be successfully adapted to microsphere format without a loss in sensitivity (generally 10's to 100's of pg/ml) or decreased dynamic range (generally 2 OOM).

The intra- and inter-assay coefficients of variation (CV) in microsphere assays were assessed using at least duplicate samples from the same plate or evaluating results of 2 or more samples from different plates/days and are presented in Figures 1B and 1C, respectively. Across all bead sets, intra-assay CVs were typically below 5%, while inter-assay CVs were somewhat higher, but were generally below 10%. These performance characteristics indicate high reproducibility, and are at least comparable with those observed in commercial microsphere assays (Staples et al., 2013). When the same samples were run in duplicate in both microsphere and ELISA format, CVs were generally consistent between methods (Figure 1D). However, the somewhat improved performance of microsphere assays for HBD2 and LL-37 resulted in statistically significant better performance across the complete set of assays when conducted in microsphere format ($p=0.043$, Wilcoxon matched pairs signed rank test).

3.2 Ability to multiplex

Because the main advantage of microsphere-based assays is the ability to assess the concentration of multiple analytes simultaneously, we next determined whether all 10 bead-based assays were compatible. We found that 8 of 10 analytes were successfully quantified when PBS-B was used as the dilution and wash buffer. However, LL-37 and HNP1-3 assays, which called for proprietary buffers, both failed to perform satisfactorily under these assay conditions. Extensive buffer scouting was conducted, and combinations of PBS, PBS-B, PBST, 10 mM acetic acid 10 mM sodium phosphate pH 7.2 and vendor supplied LL-37 and HNP1-3 buffers were tested. When LL-37-specific proprietary buffers were used for washes and preparation of the standard curve, all analytes except HNP1-3 could be measured simultaneously (Figure 2A); and when the standard curve and washes were conducted in PBST, with detection antibodies diluted in proprietary HNP1-3-specific buffer, all analytes except LL-37 could be quantified in parallel (Figure 2B). Notably, this latter buffer set also shifted the dynamic range of some assays, increasing their apparent sensitivity as can be clearly seen for HBD3 in particular.

In order to ensure that detection and capture antibodies did not interact in any way that compromised assay performance, an experiment was conducted in which each analyte was assessed singly, utilizing only one bead set, and only the matched detection reagent, with a 9-plex assay, in which all bead sets and all detection antibodies, but only one analyte was present. Figure 2C presents a representative scatterplot for a single analyte comparing the MFI of an assay conducted in single-plex and 9-plex in duplicate, demonstrating excellent agreement. Across all assays, an average Pearson correlation coefficient (PCC) of 0.95 was observed, indicating that individual assays were tolerant of the presence of additional bead sets and detection reagents. Next, as a means to determine the specificity of the capture and detection antibodies in the setting of the multiplexed assay, analyte cross-talk was assessed in two ways. First, a series of drop-out experiments was conducted, in which analytes and detection reagents were screened to assess their ability to bind to mismatched bead sets. Figure 2D presents the results of one such experiment, in which all detection reagents, and either all analytes (+), or all off-target analytes (–) were present. On average, greater than 30-fold signal:noise ratios were observed. Similarly, individual analytes were assessed for their ability to generate a signal on mismatched bead sets. Figure 2E presents results observed when the antimicrobial factor RANTES was incubated with mismatched bead sets. This pattern of reactivity was quite typical, in that only the HNP1-3 bead set registered

signal somewhat above background for a number of mismatched antimicrobial peptides. However, for a given concentration of analyte, the signal observed from mismatched antimicrobial peptides was significantly lower than the magnitude of genuine HNP1-3 signal.

3.3 Sample compatibility

The ability to quantify antimicrobial peptides present both in clinical samples and in vitro cell culture supernatants is desirable. Therefore, we next tested the ability of the microsphere assay to detect each analyte when spiked into either cell culture media (Figure 2F; PCC greater than 0.99), or cervico-vaginal lavage (CVL) fluid (Figure 2G; PCC = 0.97). Cell culture supernatants from epithelial cells from the endocervix, endometrium, and ectocervix were grown in Xvivo15 clear supplemented with 10% human serum stripped media were tested and exhibited similar performance (data not shown). Problematic matrix effects were not observed, and the agreement between expected and observed analyte concentrations was good for all samples. It is worth noting that while the 48 hrs of ex vivo cell culture performed here allowed release of antimicrobial peptides, the half-life of some peptides can be relatively short and impacted by proteases and other soluble factors that may differ among samples. Thus, as with any method aimed at quantifying labile factors, care must be taken to perform sample handling consistently, and to assess the possible role of analyte degradation.

4. Conclusion

The ability to quantify multiple antimicrobials in complex biological fluids such as CVL represents an enabling method to assess the role of these cytokines and chemokines in protecting mucosal surfaces from infection. Significantly, we found that all commercial ELISA kits tested proved amenable for adaptation to microsphere format. Unfortunately, requirements for proprietary buffers for some antibody pairs posed challenges to multiplexing, but 9 of 10 desired analytes could be assessed simultaneously. Indeed, the relative ease in which ELISA assays could be adapted to parallel multiplexed analysis points toward the possibility that additional ELISA assays could be adapted and added to the panel. Significantly, when the cost of materials used in the microsphere assay was compared to the cost of materials for ELISA kits using undiscounted, published prices for all materials and kits, the material cost to conduct all 10 assays in ELISA format was greatly reduced. This cost differential represents a significant savings, both over ELISA kits, and relative to the typical cost of commercial microsphere assays.

Thus, we anticipate that the excellent reproducibility, broad linear range, and sample and expense-sparing features of the multiplexed microsphere assay described here will facilitate studies aimed at dissecting the role of antimicrobial peptides in providing a front line defense against pathogenic infection in mucosal tissues. As examples, because cells of the mucosa have the ability to immediately respond to pathogens and act to block infection, the ability to more comprehensively profile release of antimicrobial peptides could define their role in susceptibility to sexually-transmitted infections, determine the impact of vaginal microbicides or other antimicrobials on naturally-derived defensive secretions of the FRT, delineate the role these antimicrobials play in preventing opportunistic infections, capture their variance over the course of the menstrual cycle, or permit identification of the cell and tissue types associated with their expression.

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Highlights

- ELISA assays to quantify 10 antimicrobial factors were adapted to microsphere format
- Multiple antimicrobial peptides can be quantitatively assessed simultaneously
- The microsphere assay is compatible with biological fluids and cell secretions
- Significant advantages include reduced sample volume and cost

Figure 1. Adaptation of antimicrobial factor assays to microsphere format

A. Dose-response curves for antimicrobial factors for ELISA assays (black, OD scale at right y-axis) and microsphere assays (blue, MFI scale at left y-axis). **B.** Intra-assay CVs. **C.** Inter-assay CVs. **D.** Comparison of CVs between microsphere and ELISA assays for the same sample run in duplicate (Wilcoxon matched pairs signed rank test).

Figure 2. Multiplexed assay performance

A. 9-plex assay standard curve using LL-37 dilution buffer for standards, PBS-B as detection buffer, and LL-37 wash buffer. **B.** 9-plex assay standard curve using PBS-T to dilute standards, HNP1-3 buffer for detection, and PBS-T for washes. **C.** Scatterplot comparing results of single and multiplexed assays for analyte detection. **D.** Signal observed on the RANTES bead set in the presence of RANTES analyte (+, green bar), or the presence of all off target analytes in the absence of RANTES analyte (-, black bar). **E.** Cross-reactivity profile of the RANTES analyte across bead sets. **F.** Scatterplot comparing MFI values observed for samples suspended in either PBS-B or tissue culture media (DMEM). **G.**

Scatterplot comparing observed and expected MFI values for cervicovaginal lavage (CVL) samples.

Table 1

Reagents used in ELISA and microsphere assays

Assay	Capture Ab	Analyte	Detection
Trappin/Elafin	842342, R&D Systems	842344, R&D Systems	842343, R&D Systems
CCL20/MIP3	840316, R&D Systems	840318, R&D Systems	840317, R&D Systems
HBD-2	capture Ab from kit 900-K172, Peprotech	analyte from kit 900-K172, Peprotech	detection Ab from kit 900-K172, Peprotech
SLPI	MAB1274, R&D Systems	890149, R&D Systems	AB-260-NA, R&D Systems
CCL5/RANTES	840216, R&D Systems	840218, R&D Systems	840217, R&D Systems
CXCL12/SDF-1	840931, R&D Systems	840933, R&D Systems	840932, R&D Systems
HBD-3	AHP1802, AbD Serotec	PHP211, AbD Serotec	AHP1802B, AbD Serotec
HNP1-3	HM2058B, Hycult Biotech	HC4014, Hycult Biotech	detection Ab from kit HK317-02
Lactoferrin	HM2173B, Hycult Biotech	HK329, Hycult Biotech	HP9034B, Hycult Biotech
LL-37	HM2070B, Hycult Biotech	61302, AnaSpec	HM2071B, Hycult Biotech