Journal of Immunological Methods 425 (2015) 1–9

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Statistical approaches to developing a multiplex immunoassay for determining human exposure to environmental pathogens☆

Swinburne A.J. Augustine [a,*], Kaneatra J. Simmons [a], Tarsha N. Eason [b], Shannon M. Griffin [a], Clarissa L. Curioso [c], Larry J. Wymer [a], G. Shay Fout [a], Ann C. Grimm [a], Kevin H. Oshima [a], Al Dufour [a]

[a] National Exposure Research Laboratory, U.S. Environmental Protection Agency, 26 W. Martin Luther King Drive, Cincinnati, OH 45268, USA
[b] National Risk Management Research Laboratory, U.S. Environmental Protection Agency, 26 W. Martin Luther King Drive, Cincinnati, OH 45268, USA
[c] Oak Ridge Institute for Science and Education, Oak Ridge, TN, USA

ARTICLE INFO

Article history:
Received 9 March 2015
Received in revised form 28 April 2015
Accepted 3 June 2015
Available online 9 June 2015

Keywords:
Multiplex immunoassay
Assay optimization
Design of Experiments (DOE)
Response surface methods (RSM)
Finite mixed modeling (FMM)

ABSTRACT

There are numerous pathogens that can be transmitted through water. Identifying and understanding the routes and magnitude of exposure or infection to these microbial contaminants are critical to assessing and mitigating risk. Conventional approaches of studying immunological responses to exposure or infection such as Enzyme-Linked Immunosorbent Assays (ELISAs) and other monoplex antibody-based immunoassays can be very costly, laborious, and consume large quantities of patient sample. A major limitation of these approaches is that they can only be used to measure one analyte at a time. Multiplex immunoassays provide the ability to study multiple pathogens simultaneously in microliter volumes of samples. However, there are several challenges that must be addressed when developing these multiplex immunoassays such as selection of specific antigens and antibodies, cross-reactivity, calibration, protein-reagent interferences, and the need for rigorous optimization of protein concentrations. In this study, a Design of Experiments (DOE) approach was used to optimize reagent concentrations for coupling selected antigens to Luminex™ xMAP microspheres for use in an indirect capture, multiplex immunoassay to detect human exposure or infection from pathogens that are potentially transmitted through water. Results from *Helicobacter pylori*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, and *Salmonella typhimurium* singleplexes were used to determine the mean concentrations that would be applied to the multiplex assay. Cut-offs to differentiate between exposed and non-exposed individuals were determined using finite mixed modeling (FMM). The statistical approaches developed facilitated the detection of Immunoglobulin G (IgG) antibodies to *H. pylori*, *C. jejuni*, *Toxoplasma gondii*, hepatitis A virus, rotavirus and noroviruses (VA387 and Norwalk strains) in fifty-four diagnostically characterized plasma samples. Of the characterized samples, the detection rate was 87.5% for *H. pylori*, and 100% for *T. gondii* assays and 89% for HAV. Further, the optimized multiplex assay revealed exposure/infection to several other environmental pathogens previously uncharacterized in the samples.

Published by Elsevier B.V.

1. Introduction

Conventional approaches to studying human exposure or infection from pathogens include epidemiological surveys that collect symptomatology type data and ELISAs that detect the presence of antibodies specific to pathogens. Determination of exposure or infection from pathogens using survey-based epidemiological studies can be challenging because of difficulties enrolling participants and high cost. These observational survey-based approaches may suffer from selection bias and problems of recall (Lilienfeld, 1983). Additionally, determination of the etiologic agents may be challenging because symptoms and incubation periods may not be unique to particular pathogens. Combining epidemiologic assessment with assays to measure immunological status can be very effective in determining which pathogens present exposure risks to a given population.

Recent technological advances have provided the ability to study multiple analytes simultaneously with very small sample volumes (Ellington et al., 2010; Fu et al., 2010; Choi et al., 2013). One such technology, the Luminex xMAP (Luminex Corp., Austin, TX) bead-based immunoassay is theoretically capable of multiplexing up to 500 unique assays in as little as 50 μl of sample. This is in contrast to ELISA-type antibody-based immunoassays that can measure only one analyte at a time, are time-consuming, require larger volumes of patient sample for multiple tests, and are often costly. However, well-designed immunoassays and assay optimization tests are time consuming and

☆ Notice The United States Environmental Protection Agency through its Office of Research and Development funded and managed the research described here. It has been subjected to Agency's administrative review and approved for publication. The mention of trade names or commercial products does not constitute endorsement or recommendation for use.

* Corresponding author at: 26 W. Martin Luther King Drive, Cincinnati, OH 45268, USA.
E-mail address: augustine.swinburne@epa.gov (S.A.J. Augustine).

http://dx.doi.org/10.1016/j.jim.2015.06.002
0022-1759/Published by Elsevier B.V.

costly due to the number of factors that must be managed. They require determining settings for multiple parameters and balancing sample availability with the use of expensive reagents and equipment. Hence, it is important that these assays are systematically developed to ensure they are precise and produce optimal results.

Determining key or critical assay parameters typically involve evaluating output response given changes in parameter settings one-factor-at-a-time (OFAT). In addition to being time and resource intensive, OFAT provides no means of determining or exploring important interactions between factors. As an alternative, DOE offers an efficient means of investigating the impact of changes in system parameters, exploring factor combinations and identifying parameter settings for optimizing experimental results (Montgomery, 1997; Khuri and Mukhopadhyay, 2010). DOE has effectively been used in assay development to provide critical insight for optimizing experimental conditions for applications to include the detection of anti-drug neutralizing antibodies in human serum; optimization of an automated digital microfluidic platform immunoassay; ligand-binding assay sensitivity; optimizing ELISAs; and measuring neutralizing antibodies against *Clostridium difficile* toxins (Chen et al., 2012; Choi et al., 2013; Joelsson et al., 2008; Joyce and Leung, 2013; Sitta Sittampalam et al., 1996; Xie et al., 2013).

The goals of the present study are to optimize reagent concentrations for detecting human IgG antibodies in characterized human plasma samples and determine cut-off points for the antigens in the assay using FMM (Baughman et al., 2004). Multiplex immunoassays are not as common as singleplex methods and have the additional requirement of needing multiple optimizations. To handle this complexity, we employ the use of DOE for designing and optimizing an assay for simultaneously detecting antibodies to multiple pathogens in commercial samples. The optimized settings from these tests will later be used to investigate bathing related exposures or infections from waterborne pathogens.

2. Materials and methods

Designing a Luminex-based, indirect capture, multiplex immunoassay requires determining the optimal concentrations for antigen (Ag), primary antibody (1° Ab), biotinylated secondary antibody (2° Ab), and reporters, as well as, determining the optimal pH for coupling of antigens to the microspheres. A pH of 5.0 was previously determined to be best for the coupling reactions (Griffin et al., 2011) and we found that 2° Ab concentration and streptavidin-R-phycoerythrin (SAPE) (Life Technologies, Carlsbad, CA, USA) worked best as recommended by Luminex (SAPE concentration = biotinylated 2° Ab concentration × 1.5). Preliminary experiments showed that the reagent concentrations necessary to achieve MFIs that approached the upper Limit of Detection (LoD) of the Luminex instrument (up to 30,000 MFI) tended to vary from antigen to antigen; accordingly, a range of concentrations was tested to optimize each assay (Table 1).

Multiple sets of xMAP carboxylated microspheres were obtained from Luminex Corp. (Austin, TX, USA) at a concentration of 12.5×10^6 beads/ml each. Biotin-labeled affinity purified goat anti-human IgG (λ) and rabbit anti-goat IgG (H + L) were obtained from KPL (Gaithersburg, MD, USA). Biotin-labeled donkey anti-rabbit IgG (H + L) and donkey anti-guinea pig IgG (H + L) were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

Table 1
Factor and levels for the designed experiments. These settings were developed from preliminary experiments which indicated that reagent concentrations varied from organism to organism; hence we explored a range of concentrations.

Factor		Levels								Mean
A	Ag concentration (μg/ml)	25	50	100	250					106.3
B	1° Ab concentration (μg/ml)	0	1.563	3.13	6.3	13	25	50	100	24.8
C	2° Ab concentration (μg/ml)	4	8	10	16					9.5

2.1. Two-step carbodiimide coupling of antigens to xMAP® carboxylated beads and confirmation of antigen coupling using animal-derived antibodies

The Luminex microspheres were activated and coupled as described in Griffin et al. (2011). Coupling confirmation was performed using serial dilutions of commercially available, animal-derived primary capture antibodies specific to each antigen to ensure that the beads were coupled properly and that the dynamic range of the assay could be achieved. Briefly, a working bead mixture was prepared by diluting the coupled bead stocks to a final concentration of 100 beads/μl of each unique bead set in PBS-1% bovine serum albumin (BSA). Two-fold serial dilutions of anti-species IgG primary antibody were prepared per the manufacturer's recommendations. A MultiScreen BV 96 well 1.2 μm filter plate (Millipore, Billerica, MA, USA) was pre-wet with 100 μl of PBS-1% BSA, excess buffer was removed with a vacuum manifold and 50 μl of the working bead mixture was added to each well. The serially diluted species-specific antibody was added to the beads, the reactions were mixed gently with a multi-channel pipettor, covered, and allowed to incubate in the dark, at room temperature for 30 min at 500 rpm on a VWR™ microplate shaker (Radnor, PA, USA).

The supernatant was removed by vacuum manifold, and the wells were washed twice with 100 μl of PBS–1% BSA, 0.05% Tween 20 buffer and excess buffer removed by vacuum manifold. The beads were gently resuspended in 50 μl of PBS–1% BSA buffer with a multi-channel pipettor, and 50 μl of a 16 μg/ml of biotinylated anti-species IgG secondary detection antibody was added to each well. The filter plates were covered and allowed to incubate in the dark at room temperature for 30 min on a plate shaker. After incubation, the wells were washed twice with 100 μl of PBS–1% BSA, 0.05% Tween 20 buffer and excess buffer removed by vacuum manifold. Finally, 50 μl of 24 μg/ml SAPE was added to each well, mixed gently with a multi-channel pipettor and incubated as above for 30 min and washed twice as above. Excess buffer was removed by vacuum manifold, the beads were resuspended in 100 μl of PBS–1% BSA, and the plate analyzed on a Luminex 100 analyzer.

2.2. DOE optimization of assay parameters

Design Expert 8.0.7.1 (StatEase, Inc., Minneapolis, MN, USA) was used to design singleplex experiments using antigens to *Helicobacter pylori*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, and *Salmonella typhimurium*. The concentration of Ag coupled to the microspheres, 1° Ab, 2° Ab and SAPE were used as factors. Since 2° Ab concentration and SAPE vary together, the SAPE was used as an experimental factor but eliminated as a design factor. A full factorial design was initially used to assess the impact of factor changes on MFI response for all of the pathogens, after which, response surface methods (RSM) were employed in accordance with the optimization goal of this work. RSM uses statistical and mathematical approaches to explore the functional relationship between controllable factors and output response. They are used for modeling and analyzing problems with multiple parameters, determining factor significance, developing mathematical models and optimizing system response (Montgomery, 1997). There are a number of RSM approaches to include D-optimal, central composite designs (CCD) and Box–Behnken designs (Khuri and Mukhopadhyay, 2010; Montgomery, 1997). Each can be employed in a variety of circumstances; however, we used a D-Optimal design for our studies. A D-Optimal design is a RSM developed to select design points in a way that minimizes the variance associated with the estimates of the specified model coefficients (Khuri and Mukhopadhyay, 2010).