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Research paper

Development of a multiplex microsphere immunoassay for the quantitation of salivary antibody responses to selected waterborne pathogens

Shannon M. Griffin [a,b], Ing M. Chen [c], G. Shay Fout [b], Timothy J. Wade [d], Andrey I. Egorov [d,*]

[a] Oak Ridge Institute for Science and Education, Oak Ridge, TN, USA
[b] National Exposure Research Laboratory, USEPA, Cincinnati, OH, USA
[c] Self-employed student contractor with USEPA, Cincinnati, OH, USA
[d] National Health and Environmental Effects Research Laboratory, USEPA, 109 T.W. Alexander Drive, MD 58C, Research Triangle Park, NC 27711, USA

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ABSTRACT

Saliva has an important advantage over serum as a medium for antibody detection due to non-invasive sampling, which is critical for community-based epidemiological surveys. The development of a Luminex multiplex immunoassay for measurement of salivary IgG and IgA responses to potentially waterborne pathogens, *Helicobacter pylori*, *Toxoplasma gondii*, *Cryptosporidium*, and four noroviruses, involved selection of antigens and optimization of antigen coupling to Luminex microspheres. Coupling confirmation was conducted using antigen specific antibody or control sera at serial dilutions. Dose–response curves corresponding to different coupling conditions were compared using statistical tests. Control proteins in the specific antibody assay and a separate duplex assay for total immunoglobulins G and A were employed to assess antibody cross-reactivity and variability in saliva composition. 200 saliva samples prospectively collected from 20 adult volunteers and 10 paired sera from a subset of these volunteers were used to test this method. For chronic infections, *H. pylori* and *T. gondii*, individuals who tested IgG seropositive using commercial diagnostic ELISA also had the strongest salivary antibody responses in salivary antibody tests. A steep increase in anti-norovirus salivary antibody response (immunoconversion) was observed after an episode of acute diarrhea and vomiting in a volunteer. The Luminex assay also detected seroconversions to *Cryptosporidium* using control sera from infected children. Ongoing efforts involve further verification of salivary antibody tests and their application in larger pilot community studies.
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1. Introduction

Waterborne infections can be caused by a number of bacterial, protozoan, and viral pathogens (Reynolds et al., 2008). Outbreaks of waterborne infections in the US are typically associated with treatment failures, contamination of untreated groundwater, or secondary contamination of drinking water in the distribution system (Liang et al., 2006; USEPA, 2007). Low levels of pathogens can also be present in drinking water during normal operation of public water systems. It has been estimated that millions of sporadic waterborne infections occur annually in the US (Messner et al., 2006; Colford et al., 2006). In order to reduce public health burden of waterborne infections, USEPA promulgated new regulations that imposed more stringent treatment and monitoring requirements on public water systems (USEPA, 2006a,b).

Risk assessments provide theoretical estimates of public health effects of drinking water pollution that are used in support of USEPA's rulemaking. The Safe Drinking Water Act also mandates USEPA to conduct epidemiological studies of

Abbreviations: USEPA, US Environmental Protection Agency; CCL, contaminant candidate list; SAPE, streptavidin R-phycoerythrin conjugate; MFI, median fluorescence intensity; OD, optical density; GST, glutathione *S*-transferase; VLP, virus-like particle; CI, confidence interval; CoV, coefficient of variation.

* Corresponding author. Tel.: +49 228 815 0416.
E-mail address: andegorov@gmail.com (A.I. Egorov).

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waterborne infections to verify these risk assessments (104th Congress, 1996). However, a randomized prospective intervention study in a US community did not demonstrate an association between tap water consumption and sporadic self-reported gastroenteritis (Colford et al., 2005), while earlier studies in other countries produced inconsistent results (Payment et al., 1991, 1997; Hellard et al., 2001). Using fecal sampling in such prospective studies proved to be problematic due to low rates of compliance and pathogen detection. The invasive nature of blood sampling also limited the application of serology in prospective surveys, especially in children.

Oral fluid (hereafter referred to as "saliva") is a promising alternative to serum in evaluating immune responses to common waterborne infections (Gammie et al., 2002; McKie et al., 2002). Because it is easy and safe to collect, saliva has been used in large-scale, inexpensive, cross-sectional population surveys of several infections (Morris-Cunnington et al., 2004a, b; Quoilin et al., 2007). These previous studies used monoplex salivary immunoassays. A multiplex assay capable of simultaneous quantitation of salivary antibody responses to several pathogens would be highly advantageous. In this project, a multiplex immunoassay was developed using Luminex xMAP microsphere-based technology (Luminex Corp., Austin, TX). Several pathogens transmissible through drinking water were chosen based on their importance for public health, prior research on salivary antibody responses, and availability of purified or recombinant immunogenic proteins. Selection included causative agents of chronic or latent infection (*Helicobacter pylori* and *Toxoplasma gondii*), and transient acute infection (noroviruses and *Cryptosporidium*).

2. Materials and methods

The multiplex assay to measure specific salivary antibody responses employed a mixture of different sets of microbeads with unique fluorescent characteristics, each set coupled to a specific antigen. Two versions of this assay using isotype-specific detection antibodies were developed for measuring specific IgG and IgA responses. A supplemental duplex assay with two sets of beads coupled to anti-human IgG and IgA capture antibodies was also developed to measure total salivary immunoglobulins and quantify specific antibody responses in relation to the total isotype-specific immunoglobulin content of the sample.

2.1. Saliva and serum samples

Twenty USEPA scientists donated 200 saliva samples on a weekly or monthly basis over two weeks to nine months. Saliva was collected by rubbing the gums with an Oracol sampler sponge (Malvern Medical Developments, Worcester, UK) for 1 min, separated from sponges by centrifugation, and stored at −80 °C until use. Ten volunteers also donated finger tip blood samples. Human study protocols were approved by the Institutional Review Board at University of North Carolina, Chapel Hill, operating under an agreement with USEPA. Written informed consent was acquired from all volunteers.

This project also employed de-identified human sera from individuals seropositive to *H. pylori* (gift of Dr. Harry Kleanthous, Acambis, Cambridge, MA) and sera from children diagnosed with *Cryptosporidium*, collected before and after infection (gift of Dr. Honorine Ward, Tufts Medical Center, Boston, MA).

2.2. Coupling proteins to microspheres

Proteins were conjugated to unique sets of fluorescent Luminex microspheres according to manufacturer's recommendations. Briefly, 5×10^6 carboxylated microspheres were pelleted by centrifugation and washed in 100 μL of reagent grade water. For surface activation, beads were resuspended in 80 μL of 100 mM monobasic sodium phosphate, pH 7.4 with subsequent addition of 10 μL of 50 mg/mL *N*-hydroxysulfosuccinimide (NHS; Pierce Biotechnology, Rockford, IL) and 10 μL of 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce), followed by 20 min incubation at room temperature.

Four buffers were tested for coupling proteins to microspheres: (i) 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.0; (ii) 100 mM MES, pH 6.1; (iii) phosphate-buffered saline (PBS), pH 7.4; and (iv) 100 mM NaCl, 100 mM $NaHCO_3$, pH 9.5. Activated beads were washed twice in 250 μL of coupling buffer and resuspended in 100 μL of coupling buffer. Varying amounts of each protein were added to determine an optimal coupling concentration. Total volume was brought to 500 μL with coupling buffer, followed by rotational mixing for 2 h at room temperature. Beads were then pelleted and resuspended in blocking buffer (PBS, 0.1% BSA, 0.05% sodium azide, 0.02% Tween 20, pH 7.4), washed twice and resuspended in 1 mL of the same buffer, and stored at 4 °C.

2.3. Luminex analysis

MultiScreen BV 1.2 μm filter microplates (Millipore, Billerica, MA) were washed with 200 μL of wash buffer (PBS-0.05% Tween 20) and aspirated by vacuum manifold. Microspheres were diluted to a concentration of 100 microspheres of each type per μL in dilution buffer (PBS-1% BSA). Then, 50 μL of bead mixture and 50 μL of sample (primary antibody, diluted serum or saliva) were added to wells. Plates were incubated on a shaker for 1 h at room temperature at 500 rpm, washed 3 times, and beads resuspended in 50 μL of dilution buffer. Next, 50 μL of biotinylated detection antibody in dilution buffer was added to wells, followed by incubation for 30 min at room temperature at 500 rpm and three washes. Beads were resuspended in 50 μL of dilution buffer, then 50 μL of dilution buffer with streptavidin R-phycoerythrin conjugate (SAPE; Invitrogen, Carlsbad, CA) was added to wells. After incubation for 30 min at the previous conditions and three washes, beads resuspended in 100 μL of dilution buffer were analyzed using a Luminex-100 flow cytometer at default settings. Median Fluorescence Intensity (MFI) of the reporter signal estimated from at least 100 beads of each type was used in data analysis.

2.4. Data analysis

Luminex output data were analyzed using SAS 9.2 (SAS Institute, Cary, NC). Four-parameter S-shaped weighted logistic dose–response curves (De Lean et al., 1978; Plikaytis