Cytokine 73 (2015) 84–90

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Application of multiplex arrays for cytokine and chemokine profiling of bile

Troy J. Kemp [a], Felipe A. Castro [b,1], Yu-Tang Gao [c], Allan Hildesheim [b], Leticia Nogueira [b], Bing-Sheng Wang [d], Lu Sun [e], Gloriana Shelton [a], Ruth M. Pfeiffer [f], Ann W. Hsing [g,h,i], Ligia A. Pinto [a,*], Jill Koshiol [b]

[a] *HPV Immunology Laboratory, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA*
[b] *Infections and Immunoepidemiology Branch, DCEG, NCI, NIH, Bethesda, MD, USA*
[c] *Department of Epidemiology, Shanghai Cancer Institute, Shanghai Cancer Institute, Shanghai, China*
[d] *Department of General Surgery, Zhongshan Hospital, School of Medicine, Fudan University, Shanghai, China*
[e] *Department of Epidemiology, Shanghai Cancer Institute, Shanghai, China*
[f] *Biostatistics Branch, DCEG, NCI, NIH, Bethesda, MD, USA*
[g] *Cancer Prevention Institute of California, Fremont, CA, USA*
[h] *Stanford Cancer Institute, Palo Alto, CA, USA*
[i] *Department of Health Research and Policy, School of Medicine, Stanford University, Palo Alto, CA, USA*

A R T I C L E I N F O

Article history:
Received 25 July 2014
Received in revised form 26 January 2015
Accepted 29 January 2015
Available online 2 March 2015

Keywords:
Bile
Gallstones
Cytokines
Chemokines

A B S T R A C T

Background: Gallbladder disease is highly related to inflammation, but the inflammatory processes are not well understood. Bile provides a direct substrate in assessing the local inflammatory response that develops in the gallbladder. To assess the reproducibility of measuring inflammatory markers in bile, we designed a methods study of 69 multiplexed immune-related markers measured in bile obtained from gallstone patients.
Methods: To evaluate assay performance, a total of 18 bile samples were tested twice within the same plate for each analyte, and the 18 bile samples were tested on two different days for each analyte. We used the following performance parameters: detectability, coefficient of variation (CV), intraclass correlation coefficient (ICC), and percent agreement (concordance among replicate measures above and below detection limit). Furthermore, we examined the association of analyte levels with gallstone characteristics such as type, numbers, and size.
Results: All but 3 analytes (Stem Cell Factor, SCF; Thrombopoietin, TPO; sIL-1RI) were detectable in bile. 52 of 69 (75.4%) analytes had detectable levels for at least 50% of the subjects tested. The within-plate CVs were ⩽25% for 53 of 66 (80.3%) detectable analytes, and across-plate CVs were ⩽25% for 32 of 66 (48.5%) detectable analytes. Moreover, 64 of 66 (97.0%) analytes had ICC values of at least 0.8. Lastly, the percent agreement was high between replicates for all of the analytes (median; within plate, 97.2%; across plate, 97.2%). In exploratory analyses, we assessed analyte levels by gallstone characteristics and found that levels for several analytes decreased with increasing size of the largest gallstone per patient.
Conclusions: Our data suggest that multiplex assays can be used to reliably measure cytokines and chemokines in bile. In addition, gallstone size was inversely related to the levels of select analytes, which may aid in identifying critical pathways and mechanisms associated with the pathogenesis of gallbladder diseases.
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1. Introduction

Gallbladder disease is the second highest cause of hospital admissions in the United States, with costs surpassing $2 billion [1]. Two key factors associated with gallbladder disease, gallstones and chronic inflammation, likely contribute to the hospital admissions. The chronic inflammation associated with gallstones in the gallbladder may be through the mechanical irritation of the gallstones rubbing against the gallbladder epithelial wall and gallstone-related cholestasis [2]. In addition, important risk factors

* Corresponding author at: HPV Immunology Laboratory, Frederick National Laboratory for Cancer Research/Leidos Biomedical Research, Inc. Building 469, Room 111, Frederick, MD 21702, USA. Tel.: +1 301 846 1766; fax: +1 301 846 6954.
E-mail addresses: kemptj@mail.nih.gov (T.J. Kemp), felipe.castro@nih.gov, f.castro@Dkfz-Heidelberg.de (F.A. Castro), ytgao@vip.sina.com (Y.-T. Gao), hildesha@exchange.nih.gov (A. Hildesheim), leticia.nogueira@nih.gov (L. Nogueira), wang.bingsheng@zs-hospital.sh.cn (B.-S. Wang), sunlush@126.com (L. Sun), Gloriana.Shelton@nih.gov (G. Shelton), pfeiffer@mail.nih.gov (R.M. Pfeiffer), Ann.Hsing@cpic.org (A.W. Hsing), pintol@mail.nih.gov (L.A. Pinto), koshiolj@mail.nih.gov (J. Koshiol).
[1] Present address: Clinical Epidemiology and Aging Research, German Cancer Research Center (DKFZ), Heidelberg, Germany.

http://dx.doi.org/10.1016/j.cyto.2015.01.033
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for gallstones, such as obesity, serum lipids, and diabetes have also been linked to inflammation [3–5]. Furthermore, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been associated with a reduction in gallbladder cancer [6], again suggesting that inflammation is an important mechanism in gallbladder pathogenesis.

Measurement of immune-related proteins in bile may help in characterizing the local inflammatory response that develops during gallbladder pathogenesis. However, these measurements can be challenging given the complex composition of bile, including bile salts, cholesterol, phospholipids, bilirubin, proteins, and inorganic ions (calcium, chloride, sodium, and bicarbonate) [7]. Previous studies measuring immune analytes in bile have largely been conducted in liver transplant patients where the bile was primarily collected from livers, T-tubes, or via endoscopic retrograde cholangiopancreaticography [8–16]. Also, all of these studies measured only a small number of analytes.

To facilitate future studies of inflammation and gallbladder disease, we evaluated the utility of multiplex assays to reliably measure multiple immune-related analytes in bile. Multiplex assays offer many advantages over singleplex assays such as ELISA, including (1) small sample volume requirement, (2) reduction in assay time, (3) reduced labor and material expenses, and (4) a larger range of quantification for each analyte. We have previously validated Luminex bead-based multiplex assays in serum and plasma [17], and applied these assays to identify inflammatory markers associated with risk of various types of cancer, including lung and non-Hodgkin lymphoma [18–20]. More recently, we established that it is feasible to perform these assays in cervical secretions [21], allowing future studies to evaluate the immunological associations with HPV-related cervical disease. In order to study the role of immune processes in gallbladder-related diseases, we developed methods to measure immune-related analytes in bile and examined the performance of multiplex cytokine assays in bile using samples from an NCI-sponsored study of Biliary Tract Cancers in Shanghai.

2. Material and methods

2.1. Study population

Biologic specimens and data were obtained from a population-based case-control study conducted in Shanghai, China [22]. All study participants provided written informed consent, and the Shanghai Cancer Institute and National Cancer Institute institutional review boards approved the study protocol and studies of inflammation, which includes the methods work described herein. For the assay optimization procedures, we randomly selected four gallstone patients with >1 mL of bile available, and for assessing assay reproducibility, we randomly selected 18 gallstone patients with >0.5 mL of bile available.

2.2. Optimization of immune-related analyte assays

To determine whether Luminex bead-based assays could be used to measure cytokines in bile accurately, we tested bile from four gallstone patients. The Luminex 200 instrument can detect 100 unique bead sets through a unique internal fluorescent dye signature, so the readout is highly dependent on the integrity of the fluidics system and beads. Due to the viscosity (mucins and lipids) and dark pigmentation of bile, we first evaluated whether bile would affect bead aggregation and classification. Bead aggregation ranged from 20% to 40%; however, the beads were gated to exclude doublets during acquisition.

We evaluated three methods to improve measurement reliability and bead aggregation: filtration, delipidation, and dilution. Serial filtration was performed using 1.2, 0.44, and 0.22 μm filters. Samples clogged the filters at each pore size, which minimized the utility of filtration. For delipidation, bile samples were spiked with known concentrations of several cytokines, incubated with Cleanascite™ (1:2), and treated according to the manufacture's recommendations. The removal of lipids provided minimal improvement in bead aggregation, and only three of the 19 spiked cytokines were recovered at an acceptable range (70–130%) using the Cleanascite™ reagent (Supplementary Table 1). The bile samples were serially diluted in assay buffer from the respective kit being tested, and the set of dilutions for each assay are described in Supplementary Table 1. Typically, we noticed a marked improvement in bead aggregation with diluting the bile; however, the overall improvement in bead aggregation varied with subjects. Consequently, there was a decrease in detectability at the highest dilution as compared to preceding dilutions for each multiplex panel examined, which was taken into consideration on selecting the appropriate dilution for each assay.

Furthermore, serial dilutions of bile were used to evaluate the linearity and recovery of each spiked analyte (Supplementary Table 1). We considered analytes with more than a 2-fold change between dilutions for at least half the subjects to be acceptable, except for the Milliplex soluble receptor panel, which was evaluated starting at a 1.2-fold change between dilutions. At least two concentrations of standards from each assay were spiked into the bile samples to assess recovery. A recovery of 70–130% of the spiked analyte for at least half the subjects was considered to be acceptable for bile.

2.3. Additional assays

Three analytes (C-reactive protein, CRP; Serum Amyloid A, SAA; and Serum Amyloid P, SAP) in the Luminex-based Milliplex assay (Cardiovascular Disease Panel 2) had poor spike recovery and dilution patterns for each analyte even though the linearity passed our criteria (Supplementary Table 1). Given the increasing interest in these markers, particularly CRP, in cancer, we evaluated the Vascular Injury Panel 2 (Meso Scale Discovery, MSD; Rockville, MD), which contains CRP, SAA, Intercellular Adhesion Molecule-1 (ICAM-1), and Vascular Cell Adhesion Molecule-1 (VCAM-1). Overall, the performance of the MSD Panel was better than the Milliplex panel as indicated by the acceptable linearity and spike recovery for each analyte (Supplementary Table 1), so the MSD Vascular Injury Panel was utilized for the reproducibility evaluations.

Because the concentration of bile can vary from person to person, we evaluated two different proteins that could be used as an overall measurement of bile concentration against which measurements of immune-related markers could be normalized. We examined the reproducibility of total protein (BCA, Thermo Fisher Scientific Inc., Rockford, IL), which has been used to normalize the levels of immune markers in cervical secretions [23], and albumin (Bethyl Laboratories, Inc., Montgomery, TX). The bile samples were diluted 1:100 for total protein, and 1:75,000 for albumin. The overall CV of BCA and albumin was 14.7%, and 7.9%, respectively. Additionally, hemoglobin (Bethyl Laboratories, Inc., Montgomery, TX), was used as a surrogate for contamination of bile with peripheral blood. The overall CV of hemoglobin (diluted 1:10,000) was 19.2%.

Samples undetectable at the indicated dilutions above were repeated at a different dilution for BCA ($n = 3$; 1:2), albumin ($n = 7$; 1:5000), and hemoglobin ($n = 6$; 1:20 and 1:200) in order to interpolate a detectable level based on a 5-parameter logistic curve using SoftMax Pro 6.1 (Molecular Devices, LLC., Sunnyvale, CA).

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