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Optimization and qualification of an assay that demonstrates that a FimH vaccine induces functional antibody responses in women with histories of urinary tract infections

Courtney M. Starks [a], Michelle M. Miller[b], Peter M. Broglie [b**], Joshua Cubbison [b], Steven M. Martin [a*], and Gary R. Eldridge[a]

aSequoia Sciences, Inc., 1912 Innerbelt Business Center Drive, St. Louis, MO, USA; bBioAgilytix Labs, LLC, Durham, NC, USA

ABSTRACT

Recurrent urinary tract infections (rUTI) are a serious disease associated with morbidities and mortality. Resistance to the standard of care antibiotics is now widespread because of the continued use of antibiotics among people who suffer from rUTI. We are therefore developing a vaccine to prevent recurrences among patients with rUTI. The antigen of the vaccine is FimH, a bacterial adhesin protein, and the vaccine is adjuvanted with a TLR-4 agonist. In a Phase 1 clinical study evaluating the vaccine, immunized individuals produced FimH-binding antibodies. Here we describe the optimization, qualification, and use of an assay to assess the functionality of these anti-FimH antibodies. The suitability of the assay for its intended purpose was demonstrated by selectivity, specificity, sensitivity, and intra-assay and inter-assay precision. The acceptance criteria were achieved for all parameters including intra-assay precision with ≤10% relative standard deviations and inter-assay precision with ≤25% relative standard deviations. The results presented herein suggest this functional assay will be important for supporting the vaccine's efficacy in future human studies. Furthermore and of great significance, these results prove that vaccine-induced functional antibodies can be elicited in rUTI patients against an essential virulence factor, FimH.

Introduction

According to the National Disease and Therapeutic Index (NDTI, IMS Health, Plymouth Meeting, PA) about 3 million patients experience recurrent urinary tract infections (rUTI) each year in the US.[1,2] Historically, antibiotic treatment has successfully treated individual recurrences among rUTI sufferers. But while antibiotics can treat these recurrences, they do not prevent the intense pain and anxiety felt during a UTI. In many cases, acute antibiotic regimens fail, and physicians and patients turn to prophylactic antibiotic therapy, in which antibiotics are taken daily for months at a time.[2] Not surprisingly, continued antibiotic use has led to increased antibiotic resistance among rUTI patients.[3]

New alternatives to reduce bacterial infections are desperately needed to reduce current and future morbidities and mortalities. In fact, the last new antibiotic class approved and indicated for the treatment of gram-negative bacterial infections was discovered in the early 1960s.[4–6] As a solution, experts recently called upon a global effort to develop new vaccines targeting antibiotic-resistant bacteria.[7,8]

We are developing a vaccine to prevent UTI recurrences among rUTI patients. The active immunogenic component of the vaccine is FimCH, a non-covalent complex of the bacterial adhesin protein FimH and its chaperone protein FimC. FimH

is an adhesin protein on the tip of type 1 pili, bacterial appendages that promote attachment of bacteria to host cells. Attachment occurs via branched oligomannose-containing glycoproteins that are highly conserved on the surfaces of all mammalian bladders. Type 1 pili and FimH are critical in several stages of UTI pathogenesis and are highly conserved among uropathogenic *Escherichia coli* and *Klebsiella spp*. FimH is essential for *E. coli* to colonize bladders in mice and cynomolgus monkeys, and antibodies raised against FimH antigens block the attachment of uropathogenic *E. coli* to human bladder cells.[9–13] In addition, five independent laboratories have demonstrated that immunization against FimH prevents *E. coli* from persisting in the bladders of mice and monkeys.[11,12,14–16]

Sequoia's UTI vaccine was recently evaluated in a phase 1 clinical trial to assess its safety and immunogenicity. This vaccine is adjuvanted with Phosphorylated HexaAcyl Disaccharide (PHAD®), a TLR-4 agonist. The vaccine was well-tolerated. ELISA-based immunogenicity data from the trial demonstrated that the vaccine elicited an antibody response against the N-terminus of FimH, which contains the mannose binding pocket. Elevated antibody titers, however, are not always indicative of a protective effect. Therefore, qualifying a clinically relevant and reproducible functional

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assay early in the development of a vaccine is paramount to supporting its efficacy in future human studies and proposing a correlate of protection.

A functional assay that measures the ability of anti-FimH sera from mice, rabbits, and monkeys to block binding of *E. coli* to cultured bladder cells in vitro has previously been reported.[11–13] The anti-FimH sera in these reports were obtained from animals immunized with FimCH adjuvanted with Freund's or MF59. In this published assay, bladder cells are adhered onto 96-well tissue culture plates. Fluorescently labeled *E. coli* are pre-incubated with test or control sera and then added to the wells; after washing, the level of remaining fluorescence is indicative of the level of binding of *E. coli* to the bladder cells. To determine the degree of binding specifically mediated by type 1 pili, mannose was used as a positive control; high concentrations of mannose compete for the FimH binding site and reduce binding to bladder cells. A positive control specific for type 1 pili is critical for this functional assay because depending on the strain and in vitro culture conditions, *E. coli* has dozens of adhesins that can potentially be expressed and affect reproducibility of the assay. These adhesins have been shown to bind to a variety of oligosaccharides on cultured cells and plastic surfaces.[17–21]

Herein we first describe the optimization of this functional assay to improve its reproducibility in preparation for analyzing thousands of samples from future human studies. Vaccines are typically in clinical development for ten or more years, so reproducibility of a functional assay is necessary to be able to compare results from human studies years apart. We then qualify this assay to prove its suitability to assess the functionality of vaccine-induced antibodies. We then use this qualified assay to demonstrate that antibodies induced by the FimH vaccine in humans with histories of rUTI reduce the adhesion of *E. coli* to cultured bladder cells in vitro. This is the first report demonstrating that a vaccine adjuvanted with a TLR-4 agonist elicits functional antibodies from humans against clinically relevant concentrations of *E. coli* in vitro. These results support the future evaluation of novel vaccines adjuvanted with TLR-4 agonists in humans to target gram-negative bacterial infections.

Materials and methods

Buffers and reagents

Labeling buffer contains 150 mM sodium chloride, 100 mM sodium carbonate. Pre-wash buffer contains 4% (w/v) fetal bovine serum albumin (MilliporeSigma), 1% (v/v) glycerol in phosphate buffered saline (PBS). Post-wash buffer contains 5% (v/v) fetal bovine serum in PBS. Pooled normal human serum (NHS; MilliporeSigma; 3016626) is diluted to 4% (v/v) in post-wash buffer.

Bacterial growth and labeling

E. coli J96, a uropathogenic strain from a human UTI was obtained from ATCC and stored as glycerol stocks in liquid nitrogen. Bacteria are grown and fluorescently labeled with fluorescein isothiocyanate (FITC; Sigma F7250) for each

assay. All centrifugation steps are performed at 3000 × g for 5 min at 4°C. Bacterial CFU are initially estimated using a NanoDrop One (Thermo Fisher) spectrophotometer and then confirmed by counting colony-forming units (CFU) via agar plates. Bacteria are streaked onto plates at several points during the fluorescent labeling procedure to check for contamination. Expression of type 1 pili is verified by demonstrating the mannose-sensitive hemagglutination of guinea pig erythrocytes (Colorado Serum Company, 30100).

Bacteria are grown statically by inoculating 10 mL of Luria-Bertani (LB) broth in a 50 mL flask with cells scraped from a glycerol stock with an inoculating loop. The flask is capped loosely and grown overnight at 37°C without shaking. Then, 10 μL of the culture is used to inoculate 10 mL of fresh media in a new flask; this culture is grown overnight under the same conditions and harvested by centrifugation. The pelleted bacteria are resuspended in 4 mL of labeling buffer, centrifuged, and then resuspended in 4 mL of labeling buffer. FITC (40 μL of 1% w/v in DMSO) is then added. The mixture is incubated for 1 h at room temperature in the dark, and then centrifuged. The bacterial pellet is then washed three times with PBS (Sigma P3813), removing supernatant, resuspending and centrifuging the bacteria each time. At the final centrifugation, an aliquot is removed for counting CFU via agar plates. NanoDrop is used to determine the bacterial concentration to dilute FITC-labeled bacteria in PBS to 2.5×10^8 CFU/mL the day of dilution. This bacterial stock is used to prepare the assay plates. The concentration of the bacterial stock estimated by Nanodrop is adjusted accordingly by the results of counting CFU via agar plates.

Cell culture

T24 bladder epithelial cells were obtained from ATCC and are maintained in McCoys 5A (modified) media (Gibco, 16600). For the assay, cells are washed with fresh media containing bovine serum albumin (Sigma F4135), resuspended at 4×10^5 cells/mL, and added to the wells of a 96-well flat bottom white walled tissue culture plate (100 μL/well). Cells are then allowed to adhere to the plate overnight at 37°C, 5% CO_2.

Antisera and antibodies

Characterized rabbit anti-FimH IgG reference standard

A FimCH vaccine was used to generate rabbit antiserum at Cocalico Biologicals, Inc. as previously described with the following modifications.[12] Three New Zealand white rabbits were immunized using 100 μg of FimCH that was mixed with 40 μg of phosphorylated hexaacyl disaccharide (PHAD®; Avanti Polar Lipids) in a dipalmitoyl-sn-glycero-3-phosphochiline suspension prior to each vaccination. The adjuvant suspension was prepared as detailed in Example 1 of US Patent 9,017,698.[22] The FimCH/PHAD vaccine (0.25 mL) was administered intramuscularly in two alternating sites on the hind flank on days 0, 21, 42 and 63, and exsanguination occurred on day 72. Unpurified serum from a test bleed of one rabbit at day 31 was used in development experiments. Purified IgG standard was prepared using antiserum from the exsanguination sera of the other two rabbits. IgG was purified using protein

A chromatography, and characterized using SDS-PAGE, western blot analysis, ELISA and protein concentration according to the protocol and report in Supplementary File 1. This research complied with all applicable regulations governing the care and use of laboratory animals. This study was approved by the Cocalico Animal Care and Use Committee and Sequoia Sciences' Institutional Animal Care and Use Committee.

Phase 1 human study and characterized human anti-FimH IgG reference standard

Sixty-seven adult females generally in good health were enrolled in seven cohorts in an open-label, 6-center, dose-escalating phase 1 study of the FimCH vaccine. The enrolled subjects were 18 to 64 years of age. Subjects had anti-FimH titers less than the limit of quantitation of a validated ELISA (Supplementary File 3). The first five cohorts consisted of 37 females who did not have a history of UTI. Females in cohort 1 received unadjuvanted FimCH (107 µg), and females in cohorts 2 to 5 received FimCH (50 µg) adjuvanted with either 10 µg, 20 µg, or 40 µg of PHAD®. Sixteen females were enrolled in cohort 6 and fourteen were enrolled in cohort 7, and all had a history of 5 or more documented UTI. Females in cohort 6 received 50 µg of FimCH with 40 µg of PHAD®, and females in cohort 7 received 107 µg of FimCH with 43 µg of PHAD®. Vaccinations (0.5 mL) were administered intra-muscularly in the deltoid muscle of the non-dominant arm at days 1, 31, 90, and 180. Sera were collected immediately prior to the first vaccination, and at days 31, 60, 90, 120, 180, 210, and 360. Five females in cohorts 6 and 7 left the study during the 18-month study for protocol violations as reviewed by the medical monitor. The study protocol and all amendments were reviewed and approved by Schulman Institutional Review Board and each investigator, and were allowed by the Center for Biologics Evaluation and Research, Food and Drug Administration. All subjects provided written informed consent before enrollment into the study. See the protocol in Supplementary File 2 for all inclusions, exclusions, and responsibilities of the safety review committee.

Sera from four of the subjects were selected for preparation of a human anti-FimH IgG reference standard. These subjects were enrolled in cohorts 3 and 4 and had anti-FimH titers more than 10-fold over baseline as demonstrated by a validated ELISA one month after the second or third vaccination. IgG from each antiserum was purified at American International Biotechnology Services using protein A chromatography, and then characterized using SDS-PAGE, amino acid analysis (AAA), and ELISA. Equal amounts of IgG from each of the four subjects, based on AAA, were pooled to generate a human anti-FimH IgG standard. Pooling sera from four subjects ensured that a sufficient yield of standard would be produced for use in the phase 1 study and future studies. This reference standard is 97.1% pure by SDS-PAGE, and has a protein concentration of 2.71 mg/mL by AAA. Total IgG according to reactive IgG ELISA was 4.62 mg/mL. It is a clear solution in phosphate buffered saline (pH 7.5) stored at −70°C (Supplementary File 4).

Qualified assay protocol

This procedure describes the optimized and qualified assay procedure. Parameters for method development experiments differed as described in the text and figure legends. Test and control sera or antibodies are heat inactivated for 1 h at 56°C and then diluted to 2× the final assay concentration in 4% NHS. Mannose is dissolved at 5% (w/v) in 4% NHS. FITC-labeled E. coli are diluted with PBS to 2× the final titer. A pre-incubation plate is set up with the same layout as the assay plate and loaded with 60 µL of the appropriate dilution of E. coli plus 60 µL of the appropriate dilution of serum, anti-body, or mannose. This plate is incubated for 1 h at 37°C. Meanwhile, the assay plate containing 4×10^4 T24 cells/well is washed 3× with 100 µL pre-wash buffer/well containing bovine serum albumin. The pre-incubation mixture is then transferred to the assay plate (50 µL/well), centrifuged for 1 min at $500 \times g$, and incubated for 1 h at room temperature in the dark. The plate is then washed 4× with post-wash buffer containing fetal bovine serum (100 µL/well) by gentle pipet-ting. The final wash is not removed from the wells, and the plate is read on a fluorescent plate reader (BioTek Synergy 2, BioTek Instruments) using an excitation wavelength of 485 nm and a detection wavelength of 528 nm.

Results

Initial optimization experiments of the functional assay

Initial optimization experiments focused on increasing the fluorescence signal of the bound E. coli. We tested whether a centrifugation step immediately following addition of bacteria to the cell culture plate would facilitate the reproducible adhesion of bacteria to the T24 bladder cells. A 1–3 min centrifugation step increased the signal by approximately 5- to 10-fold. This increase was not observed in wells without T24 cells, indicating that the centrifugation step did not increase nonspecific adherence of the bacteria to the plate (Figure 1A).

We then focused on understanding the fluorescence signal as a function of CFU/well binding to either 4×10^4 or 1.2×10^5 T24 cells/well. The published assay used in precli-nical studies used 4×10^4 bladder cells and 2.5×10^7 CFU of E. coli per well. We tested binding of serial dilutions of uropathogenic E. coli strain J96 (starting at 6.25×10^6 CFU/well) and found that fluorescence increased with increasing CFU/well as expected with a wide dynamic range (Figure 1B). Using 1.2×10^5 T24 cells did not increase the signal compared to using 4×10^4 cells, and so we continued to use 4×10^4 cells per well in subsequent assays.

To determine the working range of the assay specific to type 1 pili mediated binding, different concentrations of the positive control mannose were added to wells that contained 4×10^4 T24 cells and serial dilutions of E. coli. The lowest CFU/well that resulted in detectable reduction of signal in the presence of mannose was approximately 7.5×10^5 CFU/well to 1.5×10^6 CFU/well (Figure 1C). Based on these data, the lower range of the assay was set to 3.0×10^6 CFU/well. Using 6×10^6 CFU/well, replicate wells exhibited < 20% relative standard deviation (RSD), and inhibition by mannose was
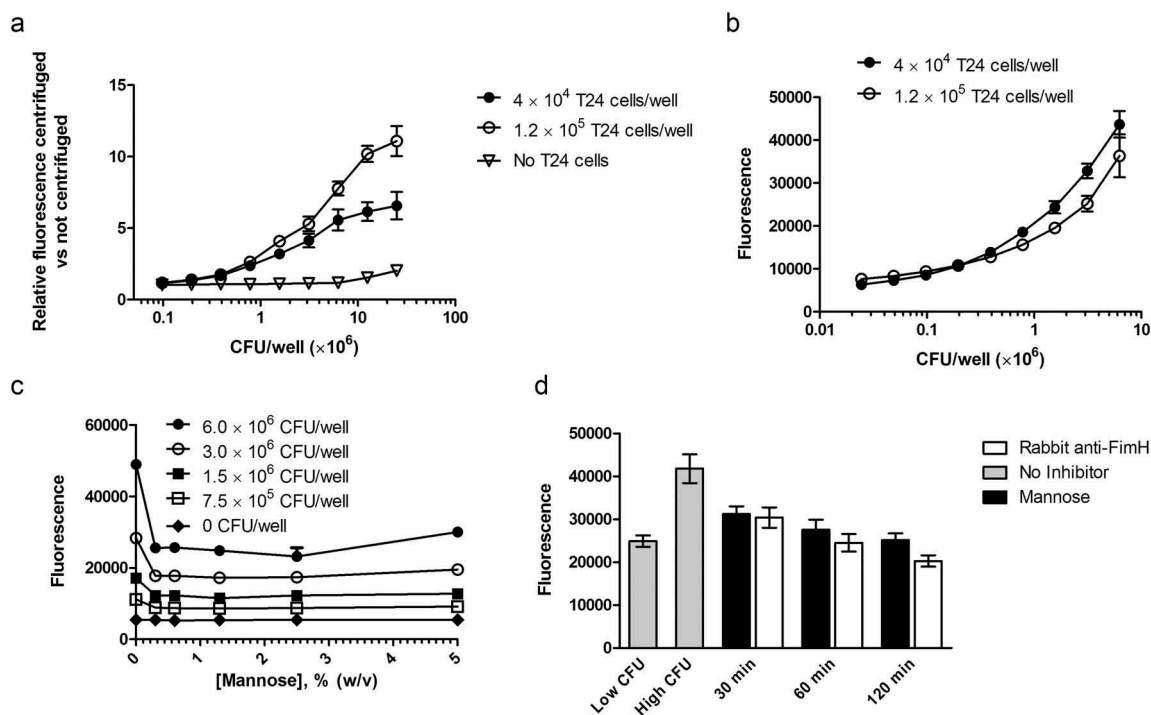
Figure 1. A. Effect of centrifugation on fluorescence. Assays were performed as described in the Methods section, except that T24 cell numbers were varied as indicated, and appropriately diluted *E. coli* was added directly to the adhered T24 cells, without a pre-incubation with test or control serum or antibodies. For each T24 cell number, two plates were prepared: one was centrifuged as described in the Methods, and one was not. Plotted here is the ratio of fluorescence from the centrifuged plate to fluorescence from the non-centrifuged plate. N = 2 wells per condition, and error bars represent standard deviation. **B**. Fluorescence signal as a function of CFU/well. Assays were performed as described in the Methods section, except that appropriately diluted *E. coli* was added directly to the adhered T24 cells, without a pre-incubation with test or control serum or antibodies. N = 6 or 12 wells per condition, and error bars represent standard deviations. **C**. Inhibition of fluorescence by mannose with varied CFU *E. coli*/well. Assays were performed as described in the Methods section, with a range of mannose concentrations. N = 2 wells per condition, and error bars represent standard deviation. **D**. Inhibition of fluorescence by mannose or rabbit anti-FimH. Assays were performed as described in the Methods section, with varied incubation time of *E. coli* with inhibitor. N = 6 (30 min and 120 min incubations) or 12 wells (controls, 60 min inubations) per condition, and error bars represent standard deviation. Two replicate plates were run on two separate days; data from one representative plate are shown.

clear, providing evidence that this level of CFU/well was suitable as the upper range of the assay. This corresponds to a bacterial cell to bladder cell ratio of 150:1, which is consistent with published methods.[11,13] Since mannose concentrations >2.5% did not further inhibit *E. coli* binding to T24 cells, 2.5% mannose (140 mM) was selected as the standard concentration for positive controls; this is also consistent with publisher reports.

We also varied the lengths of the two incubation times: pre-incubation of bacteria with serum or positive control, and incubation of bacteria with T24. Pre-incubation of bacteria with positive control or a rabbit anti-FimH serum for 0.5, 1, or 2 h gave similar results (Figure 1D); 1 h pre-incubations were adopted for subsequent assays. The majority of method development experiments used a 1 h incubation of bacteria with T24 cells; experiments with 5 or 20 min incubation did not reduce background signal (data not shown).

Analysis of rabbit and human sera and optimization of the positive and negative controls

Following the initial assay optimization, we evaluated anti-FimH serum from a rabbit immunized with FimCH adjuvanted with PHAD® on two replicate plates each day on two separate days (Figure 2A). Three dilutions of rabbit serum taken either before or after immunization were incubated with

E. coli before binding to T24 cells. Post-immune serum at 1:10, 1:20, and 1:40 dilutions reduced binding of *E. coli* to a similar extent as the 2.5% mannose positive control. Incubation with pre-immune serum resulted in fluorescence signals comparable to the high CFU/well of *E. coli*, demonstrating the selectivity of the assay.

We then examined binding inhibition by a human anti-FimH IgG reference standard diluted in normal human serum (Figure 2B). Each dilution of the human anti-FimH IgG reference standard reduced fluorescence to levels observed in the 2.5% mannose wells and the low CFU wells, indicating a decrease in binding of >3 x 10^6 CFU/well.

To address the possibility that complement-dependent cytotoxicity could interfere with the binding assay, addition of a heat inactivation step of human serum was tested. Incubation with pre-immune serum inactivated for 0, 1, or 2 h (N = 18) wells did not decrease fluorescence signals relative to the high CFU/well (Figure 2C). Similarly, heat treatment of the post-immune serum did not affect its binding inhibition. Although it appeared to have no effect, the heat treatment step was retained in the method to ensure complement activation would not affect the reproducibility of the assay in the future.

The 2.5% mannose initially chosen as a positive control typically decreased the assay signal by about 3 × 10^6 CFU/well; we therefore considered defining the signal from 2.5%
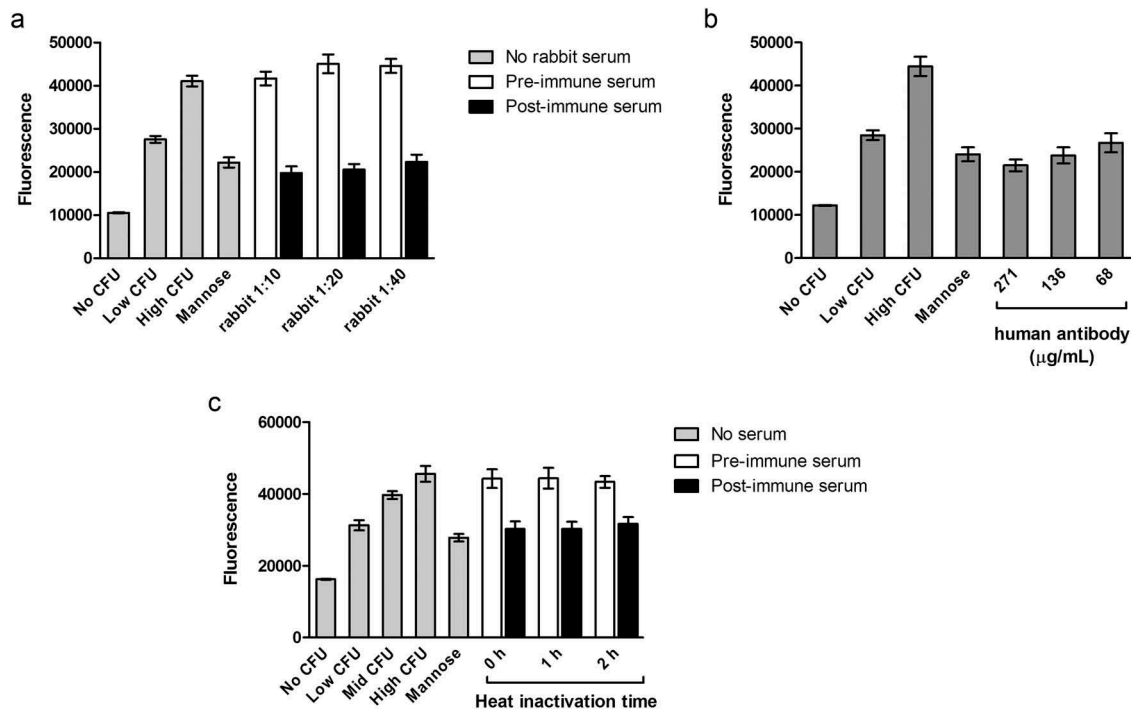
Figure 2. A. Inhibition of binding by rabbit serum. Serum was collected from a single rabbit before and 4 weeks after immunization with the FimCH vaccine. The assay was performed as described in the Methods section. Low CFU wells contained 3×10^6 CFU/well; all others contained 6×10^6 CFU/well. Two replicate plates were run on two separate days; data from one representative plate are shown. N = 6 wells per condition, and error bars represent standard deviation. The RSDs of the average fluorescence signals on each plate were ≤12%. **B**. Inhibition of binding by purified human anti-FimH IgG. Low CFU wells contained 3×10^6 CFU/well; all others contained 6×10^6 CFU/well. Two replicate plates were run on two separate days; data from one representative plate are shown. N = 6 wells per condition, and error bars represent standard deviation. The RSDs of the average fluorescence signals were <12%. **C**. Effect of heat inactivation of serum on fluorescence. Assays were performed as described in the Methods section, except that heat inactivation time varied. Low CFU wells contained 3×10^6 CFU/well, and mid CFU wells contained 4.5×10^6 CFU/well; all others contained 6×10^6 CFU/well. Serum taken from one subject before and after vaccination were assayed after 0, 1, or 2 h heat inactivation. N = 6 wells per condition, and error bars represent standard deviation.

mannose wells as 100% inhibition for the assay. However, the average signal from mannose wells compared to the average signal of the control wells containing 3×10^6 CFU/well varied by >20% week-to-week during 12 weeks of experiments. Given the heterogeneous expression of *E. coli* adhesins under phase variation, this variability is not surprising. Based on this observation and to avoid skewing the data by defining 100% inhibition with a moving target, on each plate we included 6 negative control wells that contained T24 cells plus 6×10^6 CFU/well (high CFU/well; same as in test wells), 6 negative control wells that contained T24 cells plus 4.5×10^6 CFU/well (intermediate CFU/well), 6 negative control wells that contained T24 cells plus 3×10^6 CFU/well (low CFU/well) of *E. coli*, and 6 positive control wells that contained T24 cells plus 6×10^6 CFU/well of *E. coli* plus mannose. (See Figure 4 for the plate map) Including these 24 control wells on each plate negated the variability of day-to-day *E. coli* adhesins under phase variation. The 4.5×10^6 CFU/well (intermediate) established a 50% inhibition control for each plate to control against the variable expression of adhesins. We therefore defined each dilution of a sample as passing, or positive for inhibition, if it produced an assay signal less than that of the intermediate CFU/well, which correlates to a reduction of about 1.5×10^6 CFU/well (3×10^7 CFU/mL).

Prior to qualification, human sera from three randomly selected subjects with a history of urinary tract infections from the phase 1 trial were tested according to the developed procedure (Figure 3). Sera collected pre-vaccination at day 1 and after the fourth vaccination at day 210 were analyzed on one plate per subject on two separate days. Each plate contained the 24 control wells described above. Each plate included six replicate wells of each dilution of each sample. At lease one dilution from each subject demonstrated less fluorescence than the intermediate CFU/well controls. All wells with pre-vaccination sera demonstrated equivalent fluorescence to the wells of high CFU negative control wells. All dilutions (1:20, 1:400, and 1:100) of day 210 sera from subjects 101–6004-1, 102–6005-1, and 103–6001-1 demonstrated less fluorescence than the intermediate CFU control wells the same plate, indicating a decrease in adherence of >1.5×10^6 CFU/well (3×10^7 CFU/mL). Based on the data from these samples, we adopted the plate maps in Figure 4 for analysis of additional subject sera. Subject sera were first analyzed using Plate Map 1 to screen for positive samples. Sera that tested positive at a 1:40 dilution were then further diluted and analyzed according to Plate Map 2.

Qualification

Based on method development work, the following acceptance criteria were proposed for qualification:

(1) All assay control replicates within a plate (low, intermediate, high CFU/well of *E. coli*, positive mannose control, and T24 only) must have an RSD ≤ 20%.
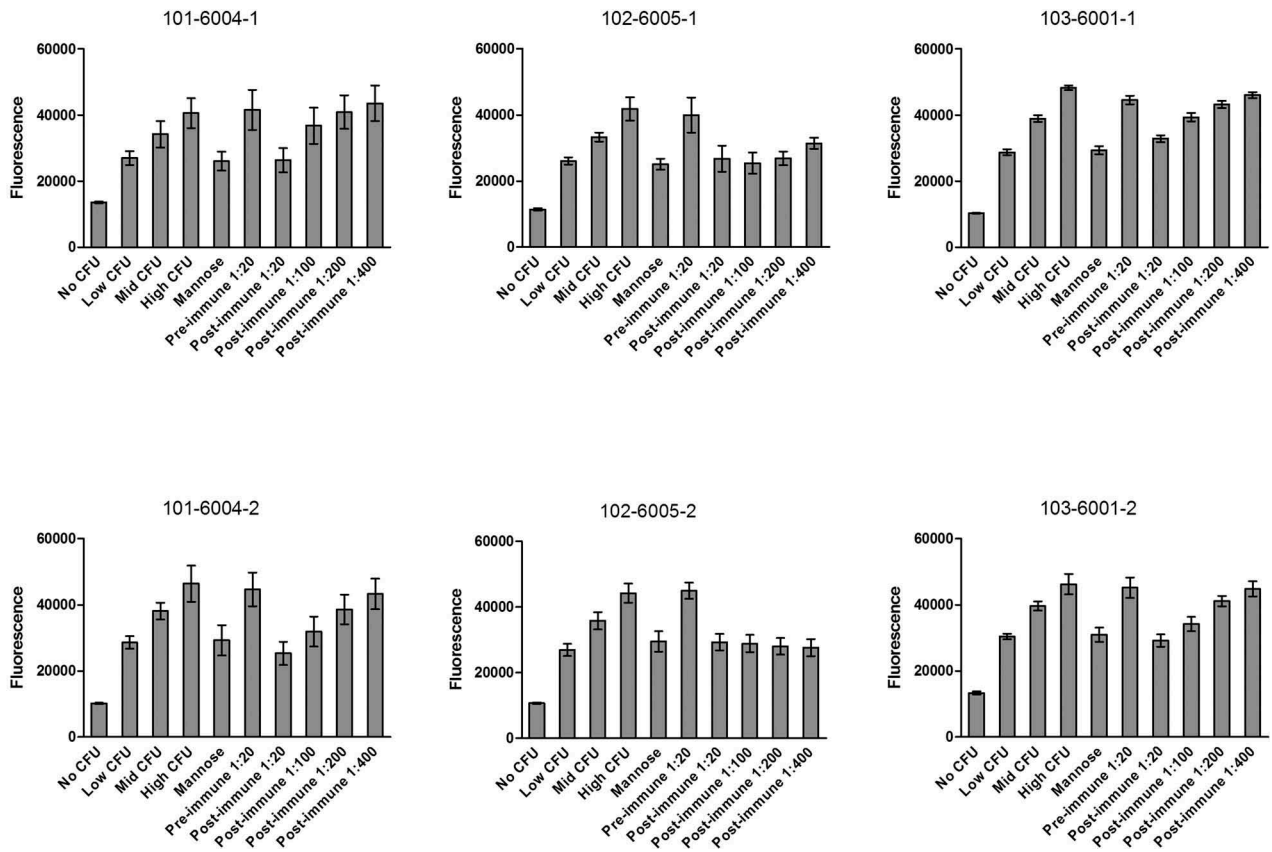
Figure 3. Inhibition of *E. coli* binding by anti-FimH sera from immunized humans with a history of urinary tract infections. Pre-immune sera were collected prior to the first vaccination, and post-immune sera were collected 30 days after the fourth vaccination. Serum from subjects 101–6004, 102–6005, and 103–6001 were each tested on two separate days as noted in the graph titles. N = 6 wells per condition, and error bars represent standard deviation.

Plate Map 1

		Blank	Low CFU Control	Mid CFU Control	High CFU Control	Mannose Control			Test wells			No *E. coli* Control	Blank
	CFU/well	0	3×10^6	4.5×10^6	6×10^6	6×10^6	6×10^6	6×10^6	6×10^6	6×10^6	6×10^6	0	0
		1	2	3	4	5	6	7	8	9	10	11	12
No T24	A	Blank	No Inhibitor	No Inhibitor	No Inhibitor	2.5 % Mannose	1:10 Pre-Immune	1:20 Pre-Immune	1:10 Post-Immune	1:20 Post-Immune	1:40 Post-Immune	Blank	Blank
	B												
	C												
4×10^4 T24/well	D		No Inhibitor	No Inhibitor	No Inhibitor	2.5 % Mannose	1:10 Pre-Immune	1:20 Pre-Immune	1:10 Post-Immune	1:20 Post-Immune	1:40 Post-Immune	T24 Only	
	E												
	F												
	G												
No T24	H		No Inhibitor	No Inhibitor	No Inhibitor	2.5 % Mannose	1:10 Pre-Immune	1:20 Pre-Immune	1:10 Post-Immune	1:20 Post-Immune	1:40 Post-Immune	Blank	

Plate Map 2

		Blank	Low CFU Control	Mid CFU Control	High CFU Control	Mannose Control			Test wells			No *E. coli* Control	Blank
	CFU/well	0	3×10^6	4.5×10^6	6×10^6	6×10^6	6×10^6	6×10^6	6×10^6	6×10^6	6×10^6	0	0
		1	2	3	4	5	6	7	8	9	10	11	12
No T24	A	Blank	No Inhibitor	No Inhibitor	No Inhibitor	2.5 % Mannose	1:40 Pre-Immune	1:80 Post-Immune	1:160 Post-Immune	1:320 Post-Immune	1:640 Post-Immune	Blank	Blank
	B												
	C												
4×10^4 T24/well	D		No Inhibitor	No Inhibitor	No Inhibitor	2.5 % Mannose	1:40 Pre-Immune	1:80 Post-Immune	1:160 Post-Immune	1:320 Post-Immune	1:640 Post-Immune	T24 Only	
	E												
	F												
	G												
No T24	H		No Inhibitor	No Inhibitor	No Inhibitor	2.5 % Mannose	1:40 Pre-Immune	1:80 Post-Immune	1:160 Post-Immune	1:320 Post-Immune	1:640 Post-Immune	Blank	

Figure 4. Plate Map 1 (upper) and Plate Map 2 (lower). Each plate contains Serum from a single patient plus bacterial controls and mannose controls.

(2) Mean signal of low CFU/well of *E. coli* < mean of intermediate CFU/well of *E. coli* < mean of high CFU/well of *E. coli*

(3) A serum sample is deemed passing (positive for inhibition) if it demonstrates a mean signal, at 1:10 or greater dilution, that is less than the mean signal of the intermediate CFU/well of *E. coli*.

(4) The lowest post-immune dilution meeting the criteria for a passing result must have a mean signal with an RSD ≤ 20%.

Assay qualification was performed to assess selectivity, specificity, sensitivity, and inter-assay and intra-assay precision.[23]

During the qualification, non-immunized sera were analyzed in 6 wells of each plate for the selectivity, specificity, sensitivity, and inter-assay and intra-assay precision experiments. In a total of 12 plates over 6 days of these qualification experiments, NHS demonstrated equivalent fluorescence signals to the high CFU/well negative controls, demonstrating selectivity of the assay (Table 1 to 4). Selectivity was also evaluated by analyzing rabbit anti-FimH IgG reference standard spiked at 100 μg/mL (final in-well concentration) into a range of dilutions of the NHS. Fluorescence signals across all spiked dilutions ranged from 28810 (NHS 1:80) to 32086 (NHS 1:20; Table 2). The difference in signals from the lowest dilution (32036 at 1:10) to the highest dilution (28949 at 1:640) was within 2 standard deviations of the means of all signals.

The specificity and relative assay sensitivity were determined by running an 8-point concentration curve of rabbit anti-FimH IgG reference standard spiked into 10% NHS (final well concentration), ranging from 300 μg/mL to 2.334 μg/mL anti-FimH IgG (Figure 5 and Table 1), according to the method. Each concentration of IgG reference standard was run in 6 wells. The RSDs of the signals of all 8 concentrations were <7%. The relative assay sensitivity was defined as the lowest concentration on the curve that produced a signal equivalent to the signal of the intermediate CFU/well control, which is the concentration of anti-FimH IgG that reduces adherence of >1.5 x 10^6 CFU/well (3 x 10^7 CFU/mL). The

assay sensitivity was determined as 20.2 μg/mL of the anti-FimH IgG reference standard.

Intra-assay and inter-assay precision were evaluated by making multiple measurements of 4% (v/v) NHS spiked with rabbit anti-FimH IgG reference standard at a final concentration of 300 μg/mL (low positive control; LPC) or 700 μg/mL (high positive control; HPC). Negative control (NC) sera consisted of 4% (v/v) NHS with no spike. As described in the methods section, these controls were prepared as 10× stocks in undiluted NHS, and then diluted at the time of the assay to mimic handling of patient serum samples. Three separate plates were run on three different days (1 plate/day) by one analyst. Each plate included 6 wells of HPC, 6 wells of LPC, and 6 wells of NC in the established assay plate format. Intra-assay precision was assessed by measuring variation in fluorescence among the six replicate wells within a plate; the HPC, LPC, and NC had <10% RSDs for nearly all three runs (Table 3). Inter-assay precision was assessed by measuring the variation in average fluorescence across the three plates; HPC, LPC, and NC had RSDs of 24.8%, 18.2%, and 3.6%, respectively (Table 4).

Analyses of sera from subjects with a history of UTI who completed the 18-month phase 1 study

The qualified assay was then used to analyze sera from twenty-six human subjects with histories of UTI who completed the 18-month phase 1 study. Sera collected prior to the

Table 1. Fluorescence as a function of concentration of rabbit anti-FimH IgG RS (reference standard) spiked into a pool of normal human sera. The full range of spiking concentrations were measured across two assay plates.

Plate 1 Sample ID	Average Fluorescence (N = 6)	%RSD
Low CFU Control	26615	5.9
Intermediate CFU Control	36114	6.2
High CFU Control	44415	5.3
Mannose Positive Control	28467	6.9
Non-Spiked NHS	44282	5.5
300 μg/mL Anti-FimH IgG RS	22167	6.6
150 μg/mL Anti-FimH IgG RS	23070	6.9
75 μg/mL Anti-FimH IgG RS	24361	4.8
37.5 μg/mL Anti-FimH IgG RS	27599	5.9
Plate 2 Sample ID		
Low CFU Control	27605	1.4
Intermediate CFU Control	37100	2.9
High CFU Control	46163	2.7
Mannose Positive Control	29433	3.4
Non-Spiked NHS	47318	3.5
18.8 μg/mL Anti-FimH IgG RS	38009	2.4
9.4 μg/mL Anti-FimH IgG RS	44644	2.5
4.7 μg/mL Anti-FimH IgG RS	47415	2.2
2.3 μg/mL Anti-FimH IgG RS	47121	2.2

Table 2. Selectivity and Specificity; rabbit anti-FimH IgG spiked at 100 μg/mL into a range of dilutions of NHS.

| Sample ID	Total Number of Replicates	Average Fluorescence	Standard Deviation	% RSD	Positive/ Negative for Inhibition
Low CFU Control	12	26971	796.8	2.8%	Positive
Intermediate CFU Control	12	36760	1290.5	3.4%	N/A
High CFU Control	12	46021	1799.3	3.7%	Negative
Mannose Positive Control	12	33016	2317.0	6.7%	Positive
1:10 NHS; Non-Spiked	6	47893	1472.1	2.8%	Negative
1:20 NHS; Non-Spiked	6	47100	1596.3	3.1%	Negative
1:40 NHS; Non-Spiked	6	47108	2540.2	4.9%	Negative
1:10 NHS; Spiked	6	32036	1684.1	4.8%	Positive
1:20 NHS; Spiked	6	32086	2010.8	5.7%	Positive
1:40 NHS; Spiked	6	30429	1888.8	5.7%	Positive
1:80 NHS; Spiked	6	28810	1744.6	5.5%	Positive
1:160 NHS; Spiked	6	29656	1979.6	6.1%	Positive
1:320 NHS; Spiked	6	29970	1707.8	5.2%	Positive
1:640 NHS; Spiked	6	28949	1924.9	6.1%	Positive
T24 Only	12	8640	170.7	1.9%	N/A
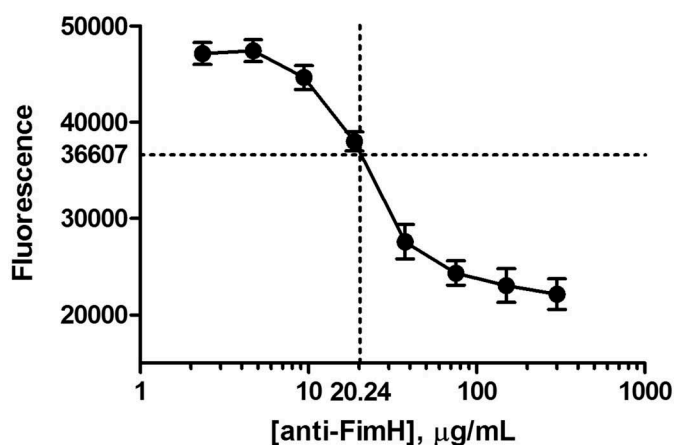
Figure 5. Fluorescence as a function of concentration of rabbit anti-FimH IgG RS (reference standard) spiked into a pool of normal human sera. Individual values are shown in Table 1.

first vaccination (day 1) and 30 days after the fourth vaccination (day 210) were analyzed. (Day 1 serum was unavailable for one subject). Twenty-one subjects had their day 1 sera tested in the assay at a 1:10 dilution and 4 subjects had their day 1 sera tested at a 1:20 dilution. Day 1 sera from 22 of the subjects did not inhibit adherence at either 1:10 or 1:20 dilution; for three subjects, a 1:10 dilution of the day 1 sera did show some apparent inhibition of adherence, with fluorescence equivalent to that of the intermediate CFU/well control. An increase in functional antibodies after vaccination was observed in 24 of the subjects for which pre-and post-vaccination sera were available. The greatest dilution of day 210 sera that reduced adherence by >1.5 x 10^6 CFU/well (3 x 10^7 CFU/mL) are shown in Table 5.

Discussion

Adherence of *E. coli* to bladder cells is the initiating event of UTI. This adherence occurs when the FimH adhesin on the surface of bacteria binds to branched oligomannose-expressing uroplakin on the surface of bladder epithelial cells. The in vitro functional assay described herein detects this binding mode to immortalized human bladder cells. Serum antibodies are evaluated against 6×10^6 CFU/well or 1×10^8 CFU/mL in this assay. Since humans experiencing UTI symptoms typically have 1×10^3 CFU/mL to $\geq 1 \times 10^5$ CFU/mL of bacteria in their urines, a reduction of binding of 3×10^7 CFU/mL of *E. coli* in this assay represents a clinically relevant result. This CFU/well is also about 2 to 3 orders of

Table 3. Intra-Assay Precision; average, SD, and RSD across replicate wells within a single plate.

Run ID	Control ID	Replicates	Average Fluorescence	SD	RSD
Run 1	HPC	6	34569	1320.4	3.8%
	LPC	6	37471	2052.8	5.5%
	NC	6	49713	1403.8	2.8%
Run 2	HPC	6	30613	2738.3	8.9%
	LPC	6	34609	3360.7	9.7%
	NC	6	46939	3481.8	7.4%
Run 3	HPC	6	20765	653.6	3.1%
	LPC	6	26010	922.6	3.5%
	NC	6	46524	1272.0	2.7%

Table 4. Inter-Assay Precision; average, SD, and RSD of results from three different plates.

Control ID	Run ID	Average Fluorescence	Inter-Assay Mean	SD	RSD
HPC	Run 1, Day 1	34569	28649	7108.6	24.8%
	Run 2, Day 2	30613			
	Run 3, Day 3	20765			
LPC	Run 1, Day 1	37471	32697	5965.4	18.2%
	Run 2, Day 2	34609			
	Run 3, Day 3	26010			
NC	Run 1, Day 1	49713	47725	1733.8	3.6%
	Run 2, Day 2	46939			
	Run 3, Day 3	46524			

Table 5. Dilutions of Sera from FimH-Immunized Humans that Inhibit the Adherence of *E. coli* to Human Bladder Cells.

Vaccine Administered	Subject Number	Sera Dilutions that Inhibited the Adherence of $\geq$1.5 x 10^6 CFU/well of *E. coli*	
		Day 1	Day 210
50 μg of FimCH with 40 μg of PHAD®	101–06-001	<10	40
	101–06-004	<20	40
	101–06-005	<10	10
	102–06-004	10	320
	103–06-001	<20	40
	105–06-002	<10	20
	106–06-001	10	160
	102–06-005	<20	320
	101–06-007	<10	20
	106–06-002	<10	80
	106–06-003	<20	20
	106–06-004	no sample	160
	101–06-008	10	640
107 μg of FimCH with 43 μg of PHAD®	106–07-001	<10	<10
	106–07-002	<10	160
	106–07-003	<10	160
	102–07-001	<10	160
	105–07-002	<10	20
	107–07-003	<10	80
	105–07-003	<10	40
	105–07-005	<10	40
	106–07-004	<10	20
	106–07-006	<10	160
	106–07-007	<10	20
	101–07-004	<10	10
	106–07-008	<10	80

magnitude greater than that typically used in opsonization functional assays for many approved vaccines.

Most importantly, the qualified assay was employed to evaluate serum antibodies elicited from women immunized with a FimH vaccine adjuvanted with a TLR-4 agonist. During these analyses, the performance of the assay was consistent with the results of the qualification. When comparing sera collected before the study to sera collected 30 days after the fourth vaccination, all subjects showed an increase in functional antibodies. These results are significant for rUTI sufferers because these women have previously been exposed to FimH and type 1 pili during their rUTI. Despite these previous exposures, a FimH vaccine adjuvanted with a TLR-4 agonist was able to induce functional antibodies that reduced binding of *E. coli* to bladder cells in vitro.

Despite years of research evaluating vaccines in animals and humans, no vaccine indicated for reducing rUTI is approved for use. A vaccine against O-antigens of *E. coli* is currently under development for extra-intestinal pathogenic *E. coli* infections, potentially including UTI.[24–26] Two

reports[25,27] demonstrate that this vaccine elicits functional antibody responses as measured by an opsonization killing assay, suggesting a different mechanism of action from the vaccine described here. No correlate of protection has been established for the O-antigen vaccine.

Vaccines typically display a correlation between antibody titer and protection from disease, but the exact correlation depends on many factors including characteristics of the patient, the vaccine, the disease, and the assays used.[28] Comparing correlates of protection among different vaccines is further complicated by the different units, not readily interconverted, for measuring the concentrations of antibody required (e.g. IU/mL, µg/mL, fractional titer of serum). Future phase 2 and phase 3 studies of our UTI vaccine will examine the correlations between total anti-FimH titer, functional responses, and rates of UTI recurrence. The assay described here is well suited to measure functional antibody responses in the range required for protection for other bacterial vaccines (e.g. 1:8 serum dilution for a pneumococcal vaccine, 1:4 serum dilution for a meningococcal vaccine), and viral vaccines (e.g. 1:4 serum dilution for hepatitis A, 1:40 serum dilution for influenza, 1:4–1:8 serum dilution for polio, and 1:20 for smallpox).[28,29]

We recognized the challenges of qualifying this assay prior to its start. The expression of type 1 pili is under phase variation, meaning that it is heterogeneously produced in E. coli populations. Therefore, even under laboratory growth conditions optimized to promote type 1 pili expression, pili expression varies day-to-day and among individual E. coli cells; individual E. coli may have 0–200 pili or more.[30] Therefore, laboratory assays investigating type 1 pili typically require dense populations of E. coli to ensure day-to-day reproducibility. We believe the 18 negative control wells per plate, 6 positive controls wells per plate, and the intermediate CFU/well control used as a cutoff for dilutions considered positive, resolved potential reproducibility issues. The intra-assay and inter-assay precision results support the reproducibility of the assay.

Another potential limitation of this assay is that E. coli has been demonstrated to bind to a variety of surfaces including polyvinyl chloride, polypropylene, polycarbonate, polystyrene, and borosilicate glass. E. coli can also bind to mammalian cells using alternative mechanisms not requiring type 1 pili; however, the medical relevance of these binding modes in UTI has not been established. This limitation of the assay was resolved by including 6 wells of mannose as a positive control on each plate to ensure that FimH-mediated binding is being measured. Furthermore, a detection method based on colony counting is not suitable for measuring thousands of serum samples from phase 2 and phase 3 human studies. The fluorescent readout of this assay is much better suited to the need for high-throughput analysis.

In summary, we optimized, qualified, and used a functional assay for measuring anti-FimH-mediated inhibition of E. coli binding to human bladder cells. This assay is designed to detect functional anti-FimH antibodies present in human serum samples after vaccination with the FimH antigen.

Abbreviations

NHS normal human serum
RSD relative standard deviation
UTI urinary tract infection
rUTI recurrent urinary tract infection

ORCID

Courtney M. Starks http://orcid.org/0000-0002-2503-8203
Peter M. Broglie http://orcid.org/0000-0003-2891-6275
Joshua Cubbison http://orcid.org/0000-0001-6585-7505
Steven M. Martin http://orcid.org/0000-0002-7832-1088

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