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UTI patients have pre-existing antigen-specific antibody titers against UTI vaccine antigens

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ABSTRACT

Urinary tract infection (UTI) is most frequently caused by uropathogenic *Escherichia coli* (UPEC). Our laboratory has been developing an experimental vaccine targeting four UPEC outer membrane receptors involved in iron acquisition – IreA, FyuA, IutA, and Hma – to elicit protection against UTI. These vaccine targets are all expressed in humans during UTI. In the murine model, high titers of antigen-specific serum IgG or bladder IgA correlate with protection against transurethral challenge with UPEC. Our aim was to measure levels of pre-existing serum antibodies to UTI vaccine antigens in our target population. To accomplish this, we obtained sera from 64 consenting female patients attending a clinic for symptoms of cystitis. As a control, we also collected sera from 20 healthy adult male donors with no history of UTI. Total IgG and antigen-specific IgG titers were measured by ELISA. Of the 64 female patients, 29 had significant bacteriuria (>10^4 cfu/ml urine) and uropathogenic *E. coli* (UPEC). Thirty-five patients had non-significant bacteriuria (<10^4 cfu/ml). Antigen-specific IgG titers did not correlate with the presence or absence of the gene encoding the antigen in the infecting strain (when present), but rather titers were proportional to prevalence of genes encoding antigens among representative collections of UPEC isolates. Surprisingly, we obtained similar results when sera from healthy male patients without history of UTI were tested. Thus, unvaccinated adults have non-protective levels of pre-existing antibodies to UTI vaccine antigens, establishing an important baseline for our target population. This suggests that a UTI vaccine would need to boost pre-existing humoral responses beyond these background levels to protect from infection.

1. Introduction

Urinary tract infection (UTI) is the second most common bacterial infection in humans [1] and half of all women will experience at least one UTI in their lifetime. Over one-fourth of those women will experience recurrent cases of UTI within one year, with almost all cases reporting very frequent UTI for the remainder of their lives [2,3]. Not only has the economic burden of treating UTI in the United States surpassed $4 billion per year, but over 4 million women suffer from chronic UTI [4,5]. At least four-fifths of these cases of acute cystitis are caused by a subset of extraintestinal pathogenic *Escherichia coli* strains (ExPEC) known as uropathogenic *E. coli* (UPEC) [6]. Acute pyelonephritis and bacteremia can also be serious complications of UTI. Indeed, the societal and economic impact of these complications caused by *E. coli,* combined with increasing antibiotic and multidrug resistance has prompted a demand for a vaccine to prevent UTI in women with a history of recurrent infection [7–9].

To identify bacterial proteins as vaccine targets against UPEC UTI infection, we previously employed a reverse vaccinology approach, using six criteria to select optimal vaccine candidates to define baseline immune responses to our antigens in our target population. These criteria included UPEC expression of the gene *in vivo* during UTI, predicted and demonstrated surface exposure of the antigen, antigenicity during experimental UTI, and gene products primarily produced by uropathogenic *E. coli* [10–19]. Our efforts to develop a UTI vaccine have focused on outer membrane siderophore and heme receptors because we found that these iron acquisition proteins fulfilled all criteria and are required for the ability of UPEC to colonize the urinary tract. Prototypic UPEC strain *E. coli* CFT073 encodes 5379 genes within its genome

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[10]. Siderophore and heme receptors satisfied the following criteria to identify vaccine candidates: (1) high *in vivo* expression in both experimentally infected mice and in women with UTI (2386 genes); (2) were predicted to be surface-exposed (343 genes) [11–13]; (3) not found in commensal strains (131 genes) (*i.e.*, pathogen-specific) [14]; (4) immunogenic during experimental infection (28 genes) [15]; (5) induced during growth in human urine (10 genes) [16]; and (6) had surface-exposed domains (8 genes) [17–19].

Using those criteria, we identified four vaccine candidates that are outer membrane iron receptor proteins, which have now been used for immunization and shown to provide protection against UPEC in CBA/J mice by reducing bacterial colonization in the bladder and the kidneys [17,18]. These four antigens have been found to be expressed by *E. coli* during human UTIs [13]. Therefore, our experimental UTI vaccine effort has focused on the following antigens: FyuA, a yersiniabactin receptor; Hma, a heme receptor; IreA, a predicted catecholate receptor; and IutA, an aerobactin receptor [17,18].

Previous studies in animal models demonstrated the potential for immunization with UPEC antigens to stimulate an effective mucosal immune response and protective serum antibody titers [17,20–23]. However, human studies with multi-strain whole cell or cell lysate UTI vaccines such as Urovac® and Urovaxom® have shown only limited long-term protection from *E. coli*-associated UTI [24–27]. Other whole-cell lysate vaccines, Urvakol® and UROstim, have not been found to significantly prevent recurrent UTI, creating a gap in the understanding of the immune responses generated by UPEC UTIs in women [8,28–31].

The goal of the present study was to define the baseline antibody responses to our four vaccine candidates in our target population prior to vaccination. Eighty-six female participants presenting with symptoms of uncomplicated cystitis at the University of Michigan Health Service Clinic in Ann Arbor, MI were enrolled in this study. Sera and infecting UTI isolates, when present, were collected from all consenting patients. In addition, sera from 20 male patients with no prior history of UTI at the University of Michigan Hospital in Ann Arbor, MI were used as controls. Existing serum antibody to experimental vaccine antigens FyuA, Hma, IreA, and IutA was measured by ELISA. The data demonstrate that most UTI patients have pre-existing antigen-specific IgG responses that represent the background antibody titers due, presumably, to previous exposure because both uropathogenic and commensal *E. coli* are known to inhabit the mammalian intestinal tract. We conclude that these levels represent non-protective background levels of these antibodies.

2. Materials and methods

2.1. ELISA: Antigen-specific IgG

Antigen-specific IgG was measured using 96-well flat-bottom plates (Corning Costar) that were prepared by first coating wells with 50 μg antigen per well in bicarbonate/carbonate buffer (3.03 g sodium carbonate, 6.0 g sodium bicarbonate per L, adjusted to pH 9.6 and stored at 4 °C). Plates were sealed and incubated at 4 °C overnight. All manipulations were performed using a BioTek microplate washer ELx405. Two-fold serial dilutions of sera were made starting at 1:400 and ending at 1:12,800 with SuperBlock Blocking Buffer in PBS (Thermo Scientific). Goat anti-human IgG-HRP (Abcam) was diluted to 1:10,000 and desired color was developed by 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Scientific). The reaction was stopped with 2 M sulfuric acid and absorbance was read at 450 nm with Instruments Gen5™ All-in-one Microplate Reader (Bio-Tek).

2.1.1. Total IgG (indirect ELISA)

Total IgG was measured using ELISA as described above, except that dilution standards were used to coat plates with either bicarbonate/carbonate-coating buffer only or with a 1:5000 dilution of bicarbonate/carbonate coating buffer and patient sera. Dilution standards were prepared using Human IgG Isotype Control (Abcam). All dilutions performed in duplicate. Patient dilutions were performed using 2-fold serial dilutions in triplicate starting at a 1:100,000 dilution with each well containing 50 μg diluted sera. Goat anti-human IgG-HRP (Abcam) was diluted to 1:1000 and measured using the above protocol.

2.1.2. Antigen-specific IgA

The protocol to measure antigen-specific IgA in human sera was the same as for antigen-specific IgG in humans with the following modifications: the secondary antibody used was Goat Anti-Human IgA alpha chain (HRP) (Abcam) at a concentration of 1 mg/ml. To make a 1:10,000 dilution, 1 μl of antibody was added to 10 mL of Superblock Blocking Buffer in PBS (Thermo Scientific).

2.1.3. Endpoint titers

Antigen-specific IgG and IgA titers in serum against each antigen were determined by ELISA as described above. The endpoint titer was defined as the reciprocal of the highest dilution that gave a positive signal at OD_{450} above no antigen control wells [32].

2.2. Commercially prepared proteins

Genes encoding the commercially prepared vaccine antigens (FyuA, IreA, IutA, Hma) were PCR-amplified from *E. coli* CFT073 (IreA, IutA, and Hma) or uropathogenic *E. coli* strain 536 genomic DNA (FyuA) and cloned into pET15b or pBAD-*myc*-HisA (Invitrogen). Recombinant protein expression from pBAD (FyuA and Hma) was induced in *E. coli* TOP10 cells to mid-log phase of OD_{600} = 0.5–1, using 100 μM L-arabinose for 4 h [17,18]. Recombinant expression from pET15b (IreA and IutA) was performed in BL21 *E. coli*. Preparation of recombinant protein from induced cultures was performed as described previously [17].

2.3. Isolation of genomic DNA and PCR

Genomic DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen) for Gram-negative bacteria. We modified the protocol by adding 2 μl RNase A. Samples were eluted in 0.1 ml aliquots with the supplied elution buffer. The concentration of purified genomic DNA was quantified and diluted to 50 ng/μl for PCR analysis. Samples were stored at −2088 °C until needed for PCR. One PCR reaction consisted of 2 μl DNA (50 ng/μl initial concentration), 1 μl Forward primer (10 μM initial concentration), 1 μl Reverse primer (10 μM initial concentration), 1 μl dNTP (2.5 mM each dNTP for 10 mM total dNTP concentration), 5 μl Taq ThermoPol Buffer (10X concentrated), 0.5 μl Taq polymerase, and 39.5 μl water. Forward and Reverse primers were designed using SeqBuilder. The annealing temperature for all PCR experiments was 55 °C, and PCR products were analyzed using 1% TAE polyacrylamide gel electrophoresis.

2.4. Blast

The NCBI-BLAST database was queried for verification of which primer sequences were affiliated with each *E. coli* UPEC UTI strain (CFT073, UTI89, and/or 536). Sixty-one samples from the BROAD database (www.broadinstitute.org) were also queried via BLAST for the presence or absence of genes encoding our four vaccine candidates.

2.5. Human subjects

Eighty-six female participants (HM01-HM86), with a median age of 21 years (age range 18–50), presenting with symptoms of uncomplicated cystitis at the University of Michigan Health Service Clinic in Ann Arbor, MI in 2012 were enrolled in this study [13]. Informed consent was obtained after the nature and possible consequences of the studies had been fully explained. Sera and infecting UTI isolates were collected from 64 consenting patients, separating each patient into one of two groups. Of the patients who consented for sera, 29 had significant bacteriuria, and all of these 29 patients had uropathogenic *E. coli* isolated from their urine and were designated as the UPEC UTI group. The other group of 35 patients had non-significant bacteriuria and only three of these patients had UPEC isolated from their urine. Sera from 20 male patients (MS01-MS20) with no prior history of UTI at the University of Michigan Hospital in Ann Arbor, MI, with an age range of 18–40, were generously provided by Dr. Ronald Giacherio and used as controls.

2.6. Statistical analysis

Comparison analyses of data were performed using Wilcoxon matched-pairs signed rank test (ELISA data) followed by Mann-Whitney tests (endpoint titers). $P < 0.05$ were considered statistically significant. Exact P values were based on two-tailed, unpaired (Mann-Whitney test) or paired (Wilcoxon matched-pairs signed rank test), nonparametric *t*-tests. All statistical tests were performed using GraphPad Prism 7.0 software.

3. Results

3.1. Study design

Eighty-six female participants presenting with symptoms of uncomplicated cystitis at the University of Michigan Health Service Clinic in Ann Arbor, MI in 2012, with a median age of 21 years (age range 18–50), were enrolled in this study (Table S1). A urine specimen was collected from all enrolled patients. Of the 86 enrolled patients, 38 had UPEC isolated from their urine. Sera was also obtained from 32 of these 38 patients with UPEC, and all but three of the 32 had significant bacteriuria (>10^4 cfu/ml urine) (Table 1). In addition, of the 86 enrolled patients, 35 patients had < 10^4 CFU/ml of urine [(No Significant Bacteriuria group (NSB)] (Table 1) with only 3 of these NSB patients having UPEC isolated from their urine specimen. All clinical isolates were analyzed for antibiotic susceptibility and identified to the species level using VITEK 2 (bioMerieux). In total, sera collected from 64 patients with either significant bacteriuria and UPEC (UPEC UTI group) ($n = 29$) or NSB ($n = 35$) (Table 1). As a control, sera were also collected from 20 men with no history of UTI.

3.2. Prevalence of vaccine antigens in UPEC isolates

We assessed the prevalence of genes encoding the four iron or heme receptors that form the basis for our experimental UTI vaccine [6,8,12,14–19,33]: *ireA, fyuA, iutA,* and *hma* by PCR. Genomic DNA was isolated from each UPEC isolate and analyzed for the presence or absence of each antigen-encoding gene by PCR. Of the 38 UPEC isolates, 31 (81%) were positive FyuA, 17 (46%) were positive for Hma, 16 (43%) were positive for IutA, and 4 (11%) were positive for IreA (Table 2). The results from this study were compared to a previous study by Spurbeck et al. [34], where 258 strains were surveyed by PCR for virulence factor genes associated with UTIs. We also queried 61 genomes from UPEC strains from *The Eli and Edythe L. Broad Institute of MIT and Harvard*'s (Broad Institute's) database. Together, we found similar prevalence data across the three studies with overall prevalence of 89% (FyuA), 62% (Hma), 51% (IutA), and 17% (IreA) (Table 2), suggesting the importance of creating a multi-subunit vaccine with a combination of the four antigens to elicit effective protection in our target population [33].

3.3. Patient antigen-specific IgG response is independent from the infecting isolate

To investigate the IgG antibody response to each vaccine antigen in both the UPEC UTI group and the NSB group, we determined both total IgG and antigen-specific IgG via ELISA. For the UPEC group, we further differentiated the ELISA data by whether the infecting isolate contained the gene encoding the antigen. We found that all the patients in this group had normal ranges of total IgG (80–280 mg/dL) (Fig. 1). We also observed that the IgG response was independent of the presence or absence of the gene in the infecting isolate. For example, 3 patients with FyuA titers of 1:12,800 represented UTIs with UPEC strains that had both presence (closed circles) and absence (open circles) of the gene encoding FyuA (Fig. 1A). Similarly, we found that patients with high antigen-specific titers to the other three antigens also represented

Table 1
Study design for collection of serum from patients with and without significant bacteriuria.

Group	Gender	Sera collected	Recurrent UTI	Non-recurrent UTI
Non-significant bacteriuria[a]	Female	35	21	14
Significant bacteriuria[b]	Female	29	19	10
Healthy controls[c]	Male	20	0	0
Total		84	40	24

[a] Patients were determined to have non-significant bacteriuria if there was <10^4 CFU/ml in urine.
[b] Patients were determined to have significant bacteriuria if there was >10^4 CFU/ml in urine. All specimens were identified as *Escherichia coli*.
[c] Patients were determined to rarely acquire a UTI.

Table 2
Prevalence of genes encoding vaccine antigens in UPEC isolates.

	FyuA	Hma	IutA	IreA	n =
Present study	81%	46%	43%	11%	38
Spurbeck et al.	90%	60–70%	40–60%	10–20%	258
Broad institute	90%	59%	57%	33%	61
Overall	89%	62%	51%	17%	357

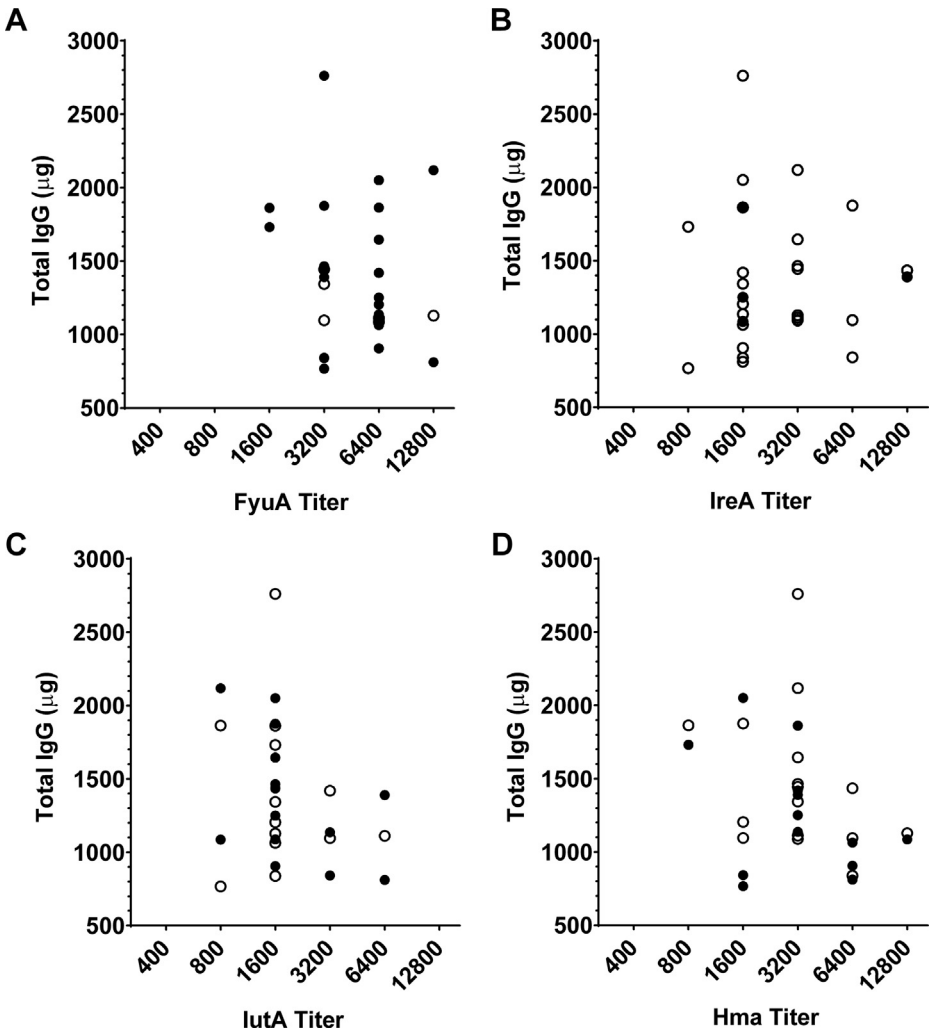

Fig. 1. Antigen-specific antibody response is independent of the presence or absence of the gene in the infecting isolate. For patients with significant bacteriuria and UPEC total IgG and antigen-specific titers for each vaccine antigen: (A) FyuA, (B) IreA, (C) IutA, and (D) Hma were determined by ELISA. Open circles indicate absence of the gene in the infecting isolate, and closed circles indicate presence of the gene in the infecting isolate. Relatively high or low total IgG did not correspond to high or low antigen-specific IgG levels, respectively. Each circle represents a single patient (n = 29).

current UTI episodes caused by strains with either presence or absence of the gene encoding the respective antigen (Fig. 1B–D). The antigen-specific titers appeared to be independent of total IgG levels since patients with higher or lower total IgG are represented across all antigen-specific titer values (Fig. 1A–D). These findings suggest that antigen-specific IgG titer is related to the general prevalence of the antigen across all UPEC isolates rather than due to the presence or absence of the gene encoding the antigen within the genome of the infecting isolate (Table S1).

3.4. Antigen-specific IgG titers are similar between patients with and without history of recurrent UTI

The IgG antibody response to each vaccine antigen was also analyzed in the NSB group and categorized by recurrent UTI history (Fig. 2). This group of patients presented with symptoms of UTI but their urine specimens had $<10^4$ CFU/ml. We differentiated the ELISA data by total IgG, antigen-specific IgG, and by patient UTI history. We found that all the patients in this group fell within normal ranges of total IgG (80–280 mg/dL) (Fig. 2). We found that in some instances, patients with recurrent UTI history (squares) had higher antigen-specific titers than patients with non-recurrent UTI history (triangles). For example, for both FyuA and Hma, we had four patient titers at 1:12,800 with recurrent UTI history, while FyuA had only a single non-recurrent patient at the highest titer and Hma had two non-recurrent patients at that titer (Fig. 2A and D). In contrast, patients had similar titers to IreA and IutA regardless of recurrent UTI history (Fig. 2B and C). Similar to what we observed for the UPEC UTI group, this group also showed higher antigen-specific titers among the most prevalent antigen, FyuA (Fig. 2A), and lower antigen-specific titers among the least prevalent antigen, IreA (Fig. 2B). Overall, there does not appear to be a strong relationship between patient UTI history and antigen-specific IgG titers (Fig. 2). Similarly, patients with UTI who also had UPEC isolated from their urine specimen did not have uniformly higher or lower antigen-specific IgG titers dependent on recurrent UTI history (Fig. S1).

3.5. UTI patients and healthy donors exhibit similar IgG responses to vaccine antigens

To better evaluate antibody responses across patient populations we compiled our ELISA data by patient status, UPEC UTI, NSB, combined UTI and NSB, and healthy donors (Fig. 3). Sera from thirty patients were analyzed for antigen-specific IgG titers against each vaccine antigen via ELISA. Six of the thirty-eight patients from

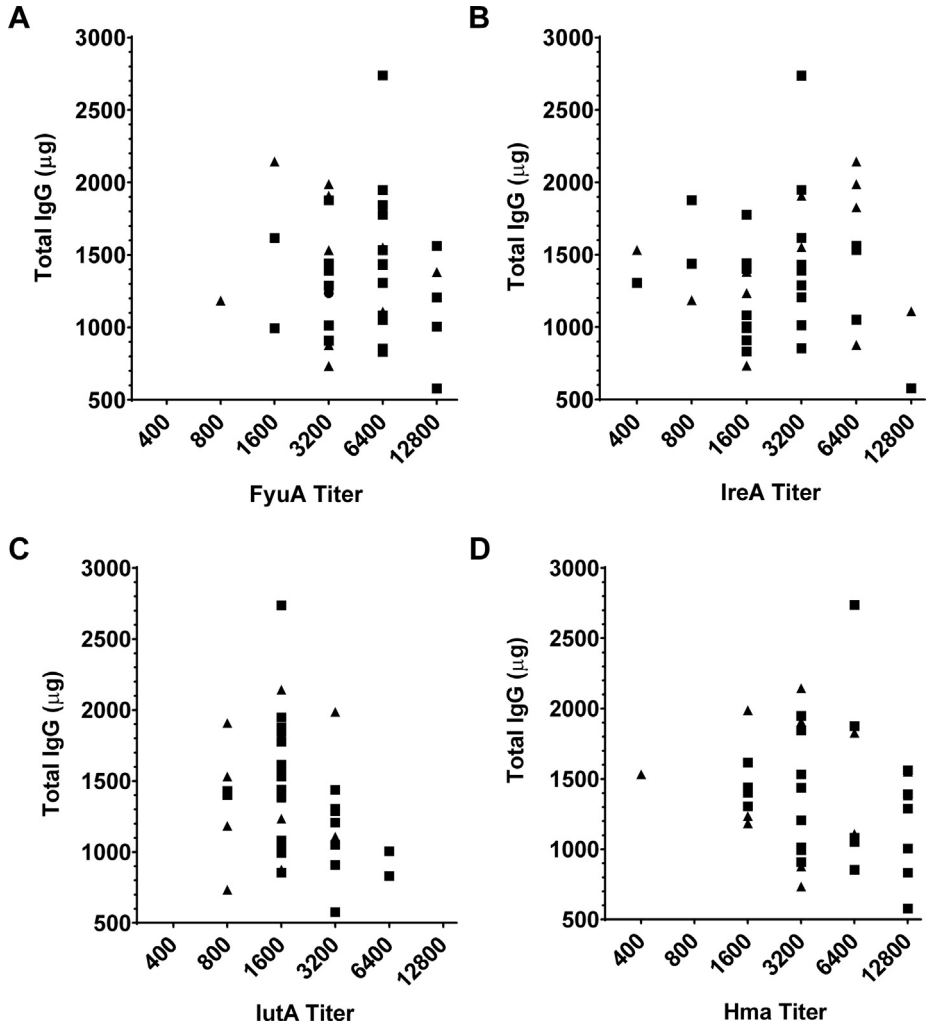

Fig. 2. Recurrent UTI history does not impact IgG titers to UTI vaccine antigens. For patients with non-significant bacteriuria total IgG and antigen-specific titers for each vaccine antigen: (A) FyuA, (B) IreA, (C) IutA, and (D) Hma were determined by ELISA. Patients with recurrent UTI history (squares) and patients with no history of UTI (triangles) are indicated for each antigen. Relatively high or low total IgG did not correspond to high or low antigen-specific IgG levels, respectively. Each point represents datum from a single patient ($n = 35$).

the clinic with confirmed UPEC UTI did not consent for serum collection and could not be included in this study. The data show the range of antigen-specific IgG titers for each of the four vaccine antigens: FyuA, IreA, Hma, and IutA. Since FyuA was the most prevalent antigen, we ran statistical analyses to determine if there was a statistically significant difference between antigen-specific antibody titers for FyuA versus the remaining 3 vaccine antigens across all cohorts (Fig. 3). Hma has a wide distribution of antigen-specific titers, whereas IutA has a narrow range of antigen-specific titers. FyuA, the most prevalent antigen, had the highest antigen-specific titers and was statistically significant when compared to IreA ($P = 0.0047$), Hma ($P = 0.0187$), and IutA ($P < 0.0001$) (Fig. 3A). These data suggest that antigen-specific IgG titer is related to the prevalence of the antigen among UPEC strains in general. Antigen-specific IgG was analyzed from patients in the NSB group as described for the UPEC UTI patients (Fig. 3B). Sera from 35 NSB patients were analyzed for antigen-specific IgG titers against each of the four vaccine antigens (FyuA, IreA, Hma, and IutA) via ELISA. The observed antigen-specific IgG responses were remarkably similar to the UPEC UTI patients. When determining if there were differences between FyuA and the other 3 antigens for this group, we found that was a statistically significant difference between FyuA and IreA ($P = 0.0021$) as well as between FyuA and IutA ($P < 0.0001$) (Fig. 3B). These data further support that antigen-specific IgG titer is related to the prevalence of the antigen in the population of all UPEC strains. We then combined the patients from the UPEC and NSB groups and found that there was a statistically significant difference between FyuA and IreA ($P < 0.0001$) and FyuA and IutA ($P < 0.0001$) (Fig. 3C). Hma had a median value of 3200, within the interquartile range of its plot. Both IreA and Hma cover the full range of titers, but IreA – the least prevalent antigen – had a lower median value of 1600 compared to that of Hma at 3200. FyuA, the most prevalent antigen, has the highest combined median titer of 6400 (Fig. 3C).

To compare a group of individuals who rarely suffer from UTI, antigen-specific IgG was analyzed from 20 male patients who had no prior history of UTI (Fig. 3D). We found that there was a statistically significant difference between FyuA and IreA ($P < 0.0001$) and FyuA and Hma ($P = 0.0007$) (Fig. 3D). These data demonstrated that healthy patients appear to have higher antigen-specific IgG titers than UTI patients, while both healthy and UTI patients have similar and normal total IgG levels. This surprising result indicates that either protected patients have protective levels of antigen-specific antibodies, thus are protected with respect to UTIs, or more likely, that these antigen-specific titers are the result of exposure to these antigens or cross-reactive antigens at sites outside of

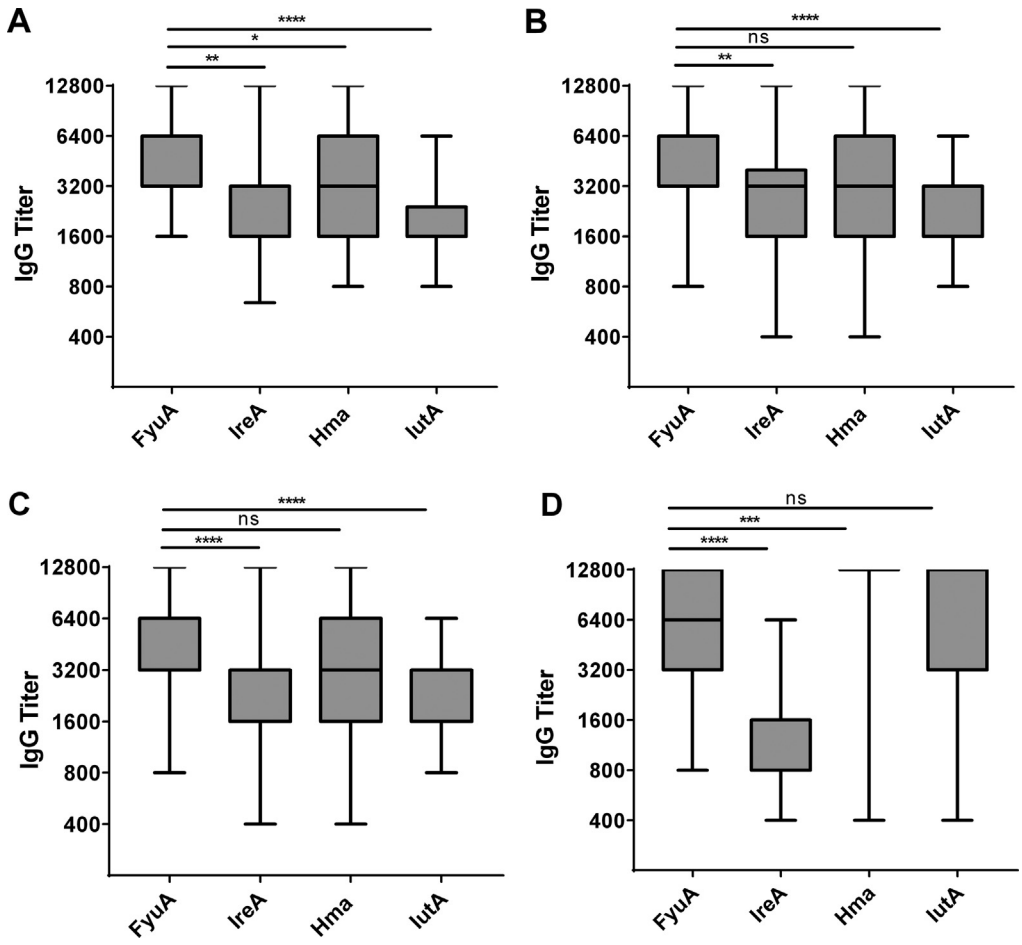

Fig. 3. All patient sera tested contain antigen-specific IgG to all four vaccine antigens. Antigen-specific titers for each vaccine antigen (FyuA, IreA, Hma, and IutA) are shown according to whether the patients had: (A) UPEC UTI episode ($n = 29$) with > 10^4 CFU/ml in urine (significant bacteriuria), (B) UTI symptoms with <10^4 CFU/ml (non-significant bacteriuria episode) ($n = 35$), (C) both groups combined (UPEC UTI episode and non-significant bacteriuria episode), and (D) healthy donors ($n = 20$), as determined by ELISA. Box-and-whisker plots represents the range of antigen-specific IgG titers for each vaccine antigen. Maximum and minimum values are represented as whiskers. Boxes represent interquartile ranges and lines represent the median. Statistically significant differences were determined by a Wilcoxon matched-pairs signed rank test; ns, $P \geq 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

genitourinary tract such as the gastrointestinal tract. Due to the similar antigen-specific responses in both the UPEC UTI and the NSB group, we decided to combine the 2 groups for additional analysis to identify potential patterns.

3.6. Patients have minimal IgA antibody response in sera

Antigen-specific IgA was analyzed from patients with various UTI histories (Fig. 4). Sera from 84 patients, including healthy controls, were analyzed for antigen-specific IgA titers against our four vaccine antigens (FyuA, IreA, IutA, and Hma) via ELISA. The median titers for both FyuA and IreA are 400, and the median titers for both IutA and Hma are 800. One patient fell below the limit of detection for FyuA and IutA, and 26 patients fell below the limit of detection for IreA. Due to technical issues with Hma, we were only able to successfully analyze titers for 41 of the patient serum samples. The patients that fell below the limit of detection were different for each antigen; no single patient fell below the limit of detection for more than one antigen. As expected, we found very low or undetectable amounts of antigen-specific IgA in serum.

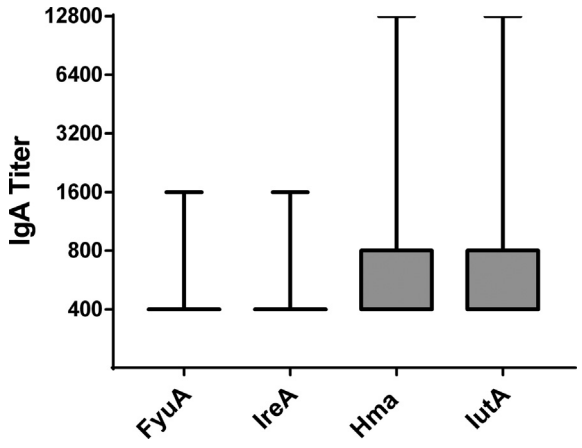

Fig. 4. Low levels of antigen-specific IgA were found in patient sera. Sera from 84 patients with various UTI histories including healthy donors without history of UTI were analyzed for antigen-specific IgA titers against four vaccine antigens (FyuA, IreA, Hma, and IutA). The box-and-whisker plot represents the range of antigen-specific IgA titers for each respective antigen. Maximum and minimum values are represented as whiskers. Boxes represent interquartile ranges.

3.7. Antigen-specific IgG titer is related to prevalence of antigen

To further explore whether or not antigen-specific serum responses are related to the prevalence of the gene encoding the antigen among UPEC strains, we looked at the percentage of patient samples occurring at each titer using our ELISA data (Fig. 5). Antigen-specific titers for all four antigens (Fig. 5) were

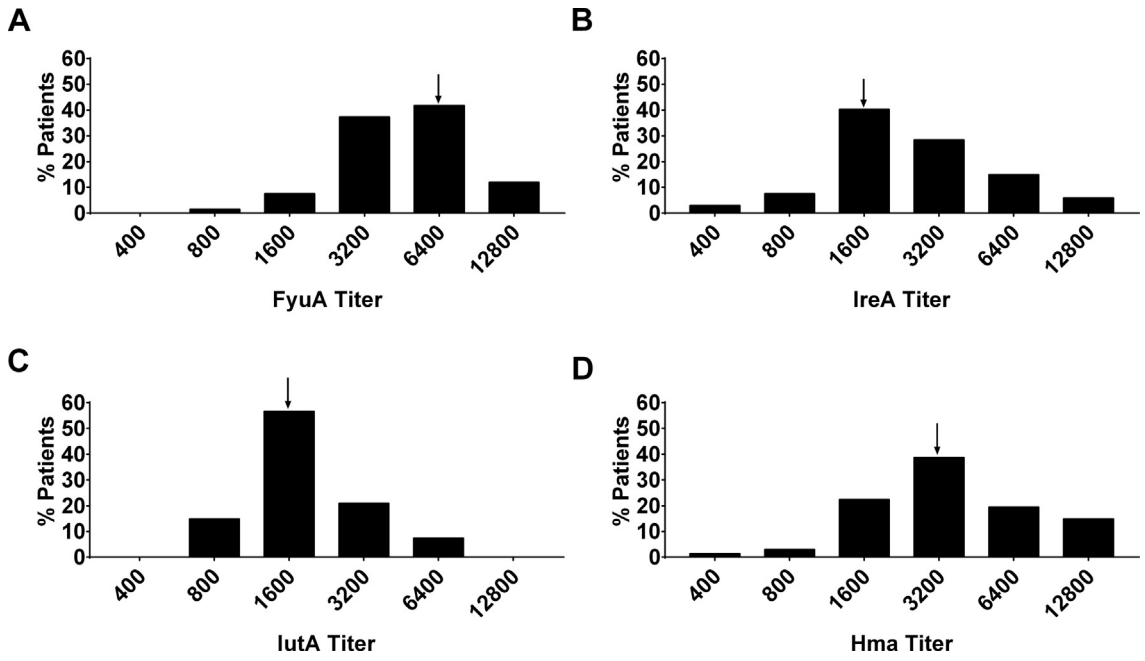

Fig. 5. Antigen-specific IgG titer is related to the prevalence of the antigen in UTI isolates. Antigen-specific IgG titers were measured by ELISA for all female patients enrolled in the University of Michigan Health Services study ($n = 64$). Antigen-specific responses were compared to percent of patient samples at each titer for each vaccine antigen: (A) FyuA, (B) IreA, (C) IutA, and (D) Hma. The median titer is indicated by a black arrow.

examined for all UTI patients for which we collected sera. FyuA is the most prevalent antigen among a representative population of UPEC strains (Table 2) and has the highest median titer (black arrow) when compared to the other three antigens: IreA, Hma, and IutA (Fig. 5A). The least prevalent antigen, IreA, has the largest percentage of patients shifted towards the lower titers (Fig. 5C). IutA and IreA have the same median titer of 1600 (Fig. 5D), and Hma has a median titer of 3200 (Fig. 5B). This supports the hypothesis that that antigen-specific titers are related to the prevalence of the antigen.

3.8. Healthy donor sera contain antigen-specific IgG

To investigate the IgG antibody response to each vaccine antigen in males who had no prior history of UTI, we determined both total IgG and antigen-specific IgG via ELISA (Fig. 6). We found that sera collected from male patients have pre-existing antigen-specific antibodies. We also found that all the patients in this group have normal ranges of total IgG (65–330 mg/dL). FyuA, the most prevalent antigen in our target population, exhibited a wide distribution of antigen-specific titers, with over half of the population at the two highest titers of 6400 and 12,800 (Fig. 6A). IreA, the least prevalent antigen in our target population, had a similar antigen-specific response in males than in our target population with a median titer of 1600 (Fig. 6B). Hma and IutA, which exhibited approximately 50% prevalence in our target population, were found to have over 50% of the population producing the highest antigen-specific response of 12,800 (Fig. 6C and D). These data suggest that samples from male patients have similar or higher pre-existing antigen-specific antibodies than our target patient population, with the possible exception of antibodies against IreA (Fig. 6B).

3.9. Patients have pre-existing antigen-specific IgG antibody responses

In total, 106 female patients and healthy male donors participated in this study and 84 of those individuals consented to donate serum samples (64 from women and 20 from men). Those samples were divided into three groups and endpoint titers were determined individually by IgG ELISA against our four vaccine antigens (FyuA, IreA, Hma, and IutA) to identify if any significant patterns exist in our cohort (Fig. 7). Of the patients with UPEC and significant bacteriuria, no significant difference was observed based on whether or not the infecting UPEC isolate contained the gene encoding the respective antigen (Fig. 7A). This finding suggests that the observed antibody responses are not elicited in direct response to the current UTI episode. There was also no significant difference observed in antigen-specific IgG titers between female patients with and without history of recurrent UTI in both the UPEC UTI and NSB groups (Fig. 7B and C). This finding indicates that recurrent UTI patients are not lacking in antibody responses to UPEC antigens or that repeated exposures to UPEC in the urinary tract do not elicit greater antibody responses than patients that may have had single or few UTI episodes. Interestingly, the only significant pattern to emerge from our study is that healthy male donors did have titers different from our combined patient groups (UPEC UTI and NSB) (Fig. 7D). Healthy controls had a significantly lower endpoint titer for IreA ($P = 0.0029$) but significantly higher endpoint titers for Hma ($P < 0.0001$) and IutA ($P < 0.0001$) when compared to all of the female patients. There was no statistically significant difference between females and healthy donors for FyuA (Fig. 7D). These data show that the individuals in our study have been exposed to these antigens and this exposure likely occurs outside of the urinary tract.

4. Discussion

Urinary tract infection (UTI) is the second most common infection in humans after respiratory tract infections [35]. This results not only in huge annual economic costs, but in decreased workforce productivity, and high patient morbidity [4]. Most infections are caused by UPEC where alarming rates of antibiotic resistance are rising [36]. Combined with increasing antibiotic resistance, allergic reactions to antibiotics and failure to prevent recurrent infections are additional barriers to treatment [36,37]. We have been developing vaccination as one approach to prevent and treat

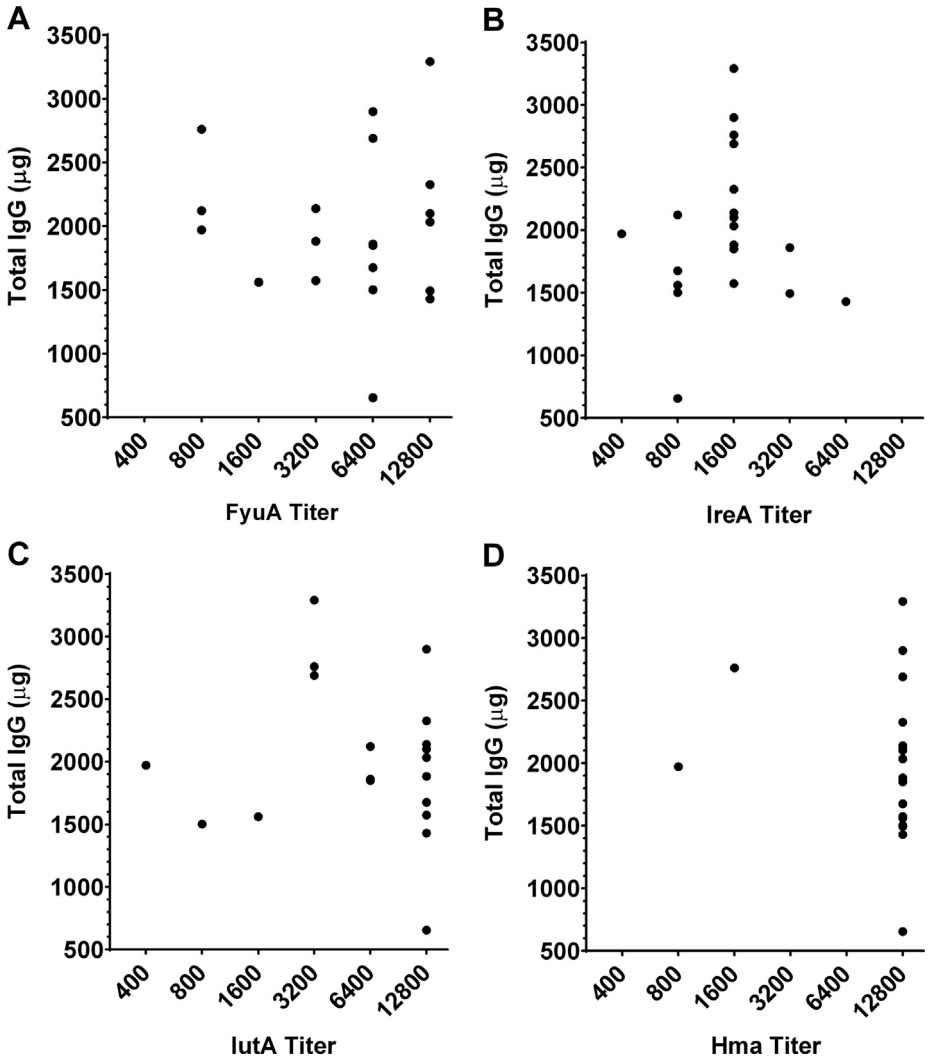

Fig. 6. Sera from healthy donors least likely to have had a UTI episode possess antigen-specific antibodies. Twenty serum samples were acquired from healthy male donors ages 18–40 without symptoms or history of UTI at the University Hospital in Ann Arbor, MI. Total IgG and antigen-specific titers for each vaccine antigen; (A) FyuA, (B) IreA, (C) IutA, and (D) Hma were determined by ELISA. Each circle indicates one patient.

these prevalent infections. Our previous work has led us to target four antigens, FyuA, IreA, IutA, and Hma, all involved in iron acquisition [17,19,38] and expressed during human UTI [6,12,13] for development of a preventive multi-subunit vaccine against UPEC. One important gap in this work that this current study addresses is to define the baseline antigen-specific antibody responses in our target patient population. The rationale for this is that we have previously demonstrated for all four vaccine antigens that antigen-specific serum IgG represents a strong correlate of protection in vaccinated mice and that high antibody titers correlate with low CFUs of UPEC following transurethral challenge of vaccinated mice with a UPEC strain [17,18,20–22].

In addition to examining antigen-specific IgG levels in UTI patients and healthy donors, we also examined genomic DNA extracted from the patient's UPEC isolates by PCR for presence or absence of genes encoding the four vaccine antigens. Previous phylogenetic analyses demonstrated that these isolates belong to two *E. coli* phylogroups most likely to represent UPEC strains, namely B2 and D. We found that FyuA was the most prevalent and IreA was the least prevalent of the four antigen-encoding genes. This was further supported by expanding the genomic data set to include 319 UPEC genomes within various databases and from previous studies, where we found the prevalence of these four genes was similar to that observed in the patient isolates from our study (Table 2). We initially set out to identify any patterns behind the levels of IgG in sera. For example, did recurrent UTI patients have higher or lower antibody titers than first-time UTI patients or do patients whose infecting isolate lack the gene encoding the antigen have lower titers to the respective antigen? Interestingly, the only pattern that emerged from the present study was that generally, patients had higher IgG titers to the antigens that are more prevalent across the population of UPEC isolates. This pattern held even for healthy male donors between the ages of 18–40, a group least likely to have UTIs. This important finding raises the notion that humans are likely exposed to these antigens or cross-reactive antigens at sites outside of the urinary tract such as the gastrointestinal tract. These findings also suggest that both the observed IgG titers are not high enough to be protective and that a multi-subunit vaccine might be useful to boost these baseline levels to protective levels as was observed experimentally in the murine model of ascending UTI.

Importantly, our study also furthers the notion that that vaccination with a single antigen would not be sufficient to protect against UPEC UTI, since no antigen is found with 100% prevalence in every UPEC strain (Table 1). In addition, it is possible that even strains that carried a gene for one of these antigens, as detected by

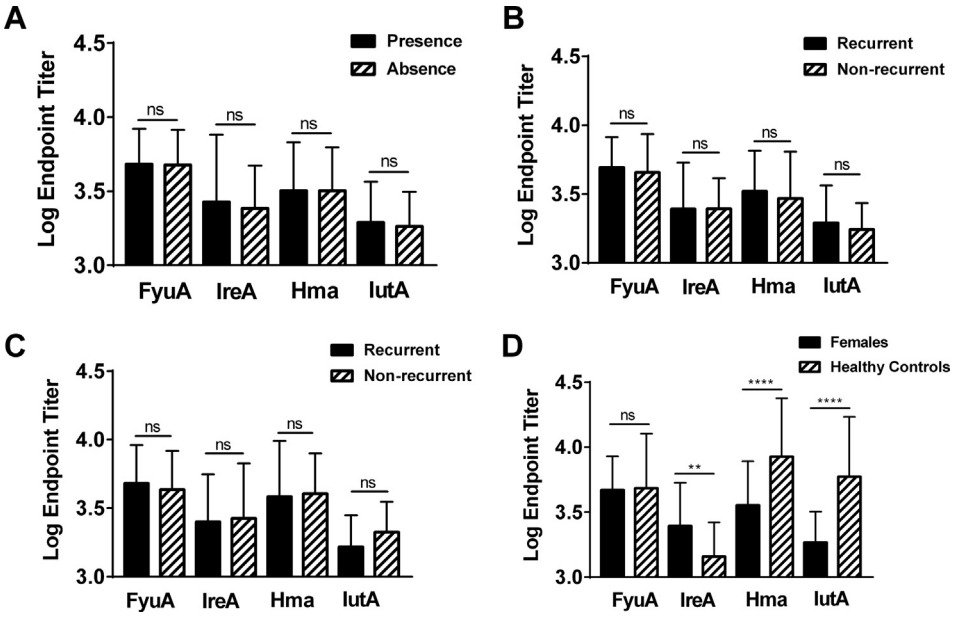

Fig. 7. Total IgG responses across all patient cohorts are similar. Endpoint titers for each vaccine antigen (FyuA, IreA, Hma, and IutA) are grouped by patients with: (A, B) a UPEC UTI episode with >10^4 CFU/ml in urine (significant bacteriuria) (n = 29), (C) a non-significant bacteriuria episode with <10^4 CFU/ml in urine (n = 35), and (D) comparison of female patients from (A–C) to healthy donors (n = 20), as determined by ELISA. In (A–C) patients were further characterized based on whether: (A) the patient UPEC isolate with presence or absence of the gene encoding the antigen, or in (B, C) the patient had recurrent or non-recurrent UTI history. Error bars represent the mean and SD values from each group. Statistically significant differences were determined by Mann-Whitney test; ns, $P \geq 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

PCR, may not express the antigen due to a deletion or insertion mutation. For example, the *fyuA* gene is present in UPEC strain CFT073, yet the strain does not express the protein due to an insertional mutation within the yersiniabactin biosynthetic operon.

Our studies focused on the levels of IgG antibodies present in the serum of each individual; however, we wanted to investigate what antibody titers could be found in the urinary tract itself. IgA is the most abundant antibody present in the urinary and gastrointestinal tract tracts. Unfortunately, we did not have sufficient urine from every patient in our study to provide a complete analysis. So, although we saw low IgA titers in peripheral blood, it remains possible that levels of secretory IgA in patients would reveal important patterns that were not discernible in our study. Furthermore, patients who are least likely to contract UTI have relatively high antigen-specific responses, suggesting that they may have encountered pathogens expressing conserved antigens similar to those expressed by *E. coli*. A vaccine to treat UTI would likely have to boost these pre-existing background responses to be effective.

5. Conclusions

The findings in the present study give us the first clear look at baseline antibody titers in the exact target patient population who would benefit from a UTI vaccine. These findings represent compelling evidence that a multi-subunit vaccine will be required to boost the observed baseline levels to titers that are protective against infection. In support of this, we know that unrelated exposed antigens from UPEC such as PapDG, hemolysin, Dr fimbria, and IroN have been shown to induce at least some immune response in immunized animals [17,39–42], however, these UTI vaccines did not reliably induce high titers of serum IgG and protection was limited in immunized animals. Since we observed remarkably stable antigen-specific titers in our target population and in healthy donors, it is not unreasonable to speculate that our multi-subunit approach could generate protective titers in a broad population of patients susceptible to UTI caused by UPEC.

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Conflict of interest

Authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.031.

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