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Iron Receptor Antigen-Based Mucosal Immunization Prevents Urinary Tract Infection

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Abstract

Urinary tract infection (UTI) is a leading bacterial infection worldwide caused by Uropathogenic *Escherichia coli* (UPEC). Despite the apparent ease of treatment, they carry an enormous health and economic burden because of the extent of relapses and resistance to anti-infective agents. This study aimed to develop a UTI vaccine that targets the iron acquisition systems of UPEC. With the help of functional vaccinology, six outer membrane iron receptors (ChuA, Hma, Iha, IreA, IroN, and IutA) were selected as possible vaccine candidates for further studies. Mouse models were immunised intranasally with recombinant antigens Hma, IreA, and IutA, mixing cholera toxin as an adjuvant. The humoral and cellular immune responses raised in vaccinated mice were highly robust. Immunisation conferred protection in specific locales, such as Hma to the kidney and IreA and IutA gave immunity to the bladder. This study is the first to describe specific immune protection against UPEC in a mouse model, as characterised by significant increases in antibody IgA in urine and IgG in serum, as well as the proinflammatory cytokines IL-17 and IFN-γ. This study provides proof of principle for iron acquisition pathways to be targets for vaccine development against UTIs. As iron is an essential nutrient for UPEC, vaccines directed at iron acquisition systems provide an excellent basis for future multivalent subunit vaccines that can alleviate the burden of UPEC infections.

Introduction

Urinary tract infection is considered among the most common bacterial infections in the world, dramatically affecting public health and inflicting a reasonably substantial economic burden. In their lifetime, more than 50% of women (53%) and about 14% of men will have had one urinary tract infection (UTI) **[1]**. In the United States alone, UTIs lead to an estimated 6.8 million physician visits, 1.3 million emergency room visits, and about 245,000 hospitalizations per year, costing over $2.4 billion altogether. Such a large figure highlights the need for more efficient preventive and therapeutic modalities **[2]**.

Escherichia coli accounts for over 80% of uncomplicated urinary tract infections. It occurs almost

entirely in patients with urinary tracts free of structural abnormalities or inflammatory lesions **[3]**. Symptoms of urinary tract infections range from acute cystitis, with dysuria and frequency, to pyelonephritis, often with systemic symptoms such as fever and flank pain. The effects may compromise much more than the immediate symptoms of the urinary tract infection; severe complications can arise, particularly among vulnerable populations **[4]**. Upper urinary tract infections in children may lead, for instance, to long-term damage to kidneys since renal scarring has been reported in about 57% of pediatric cases of acute pyelonephritis **[5]**.

Current treatments for UTIs are mainly focused on antibiotics like trimethoprim-sulfamethoxazole and ciprofloxacin. However, these antibiotics are losing their efficacy with the prohibitive rise of antimicrobial resistance **[6]**. Increasing resistance rates have been documented for uropathogenic E. coli (UPEC), making treatment difficult and posing a risk of recurrent infection. Recurrence presents a considerable challenge, as recurrences within six months of an initial infection were reported by 27% of women, with 2.7% reporting a third episode within that same time **[7]**. Re-infections often involve the same bacterial strain, with recurrence rates varying among studies from 25% to 100%. Such patterns suggest that the urinary tract may act as a reservoir for subsequent infection, adding difficulty to long-term management and highlighting the urgent need for alternative preventive measures **[8]**.

In this context, a UTI vaccine holds promise. An effective UTI vaccine may shorten the long-term morbidity and economic impact of UTIs due to long-lasting protective immunity and reduced antibiotic use **[9]**. Early vaccine development focused on bacterial capsular polysaccharides, stick LPS, and heat-inactivated bacterial preparations to galvanise protective immune responses. Although some of these approaches might have shown success in generating some level of immunity, their short-lived effects and relatively weak efficacy demanded that better approaches be developed **[10]**.

Recent advances have magnified efforts toward subunit vaccines targeting virulence factors, particularly bacterial fimbriae. Fimbriae are surface structures that have a critical role in the adherence and colonisation of the urinary tract by UPEC. For example, preclinical immunisation studies with the type 1 fimbrial adhesin FimH conjugated to its chaperone FimC have shown promise **[11]**. In murine models, the vaccine achieved a 99.9% reduction in bladder colonisation; a similar level of efficacy was present in primate studies **[12]**. Other subunit vaccines provide variable protective immune responses in tests, including P-fimbriae, alpha-hemolysin, salmochelin receptor IroN, and conjugates composed of capsular polysaccharide K13 with diphtheria toxoid **[13]**. Despite notable progress, no vaccines against UTI have been approved for clinical use in the United States-merely reflecting the challenges faced in translating preclinical triumphs into clinical application.

Recent approaches of large-scale reverse vaccinology offer a potentially powerful alternative to the classical vaccine development. These strategies exploit genomic and proteomic data to identify novel vaccine targets systematically. In UPEC, iron acquisition systems have been highlighted as one of the more prominent components supporting bacterial survival and pathogenesis. The urinary tract is an environment limited by conditions due to the need for iron. UPEC relies on specialised outer membrane receptors for iron acquisition. These include siderophore and heme receptors that confer bacterial fitness for growth and virulence. Deletions of genes encoding iron receptors, IreA, ChuA, Hma, Iha, IroN, and IutA, indeed damaged UPEC colonisation and survival in the urinary tract **[14]**.

Methodology

Bacterial Strains and Culture Conditions

This study used uropathogenic *Escherichia coli* (UPEC) CFT073 strain isolated from a patient with acute pyelonephritis. The strain was chosen for its pathogenic relevance in urinary tract infections. The bacteria were grown in Luria broth with 100 mg/mL of ampicillin shaking at 37 °C. The bacteria were initially grown to help with growth and maintenance for subsequent experiments.

Animal Model and Vaccination Strategy

The ascending UTI model in 6-8-week-old female CBA/J mice was used to investigate the efficiency of mucosal immunization. The mice were divided into experimental and control groups. The experimental group was subjected to intranasal immunizations with purified outer membrane iron receptor antigens coupled to cholera toxin CT as a mucosal adjuvant. Each mouse was administered CT linked to 100µg of antigen for the first immunization plus two booster doses of 25µg antigen linked to 2.5µg of CT at day 7 and day 14. The control group received CT without antigens. Immunizations were administrated in 20µl volume through both nares.

Antigen Selection, Preparation, and Purification

This article evaluated the six antigens for iron receptors (ChuA, Hma, Iha, IreA, IroN, and IutA) for their possible use as vaccine candidates. These proteins have been selected because they are involved in the iron acquisition of the bacteria, which is relevant for UPEC as they have to face an iron-limited environment in the urinary tract. The genes encoding these antigens were amplified from CFT073 genomic DNA using PCR-based techniques and cloned into expression vectors. The recombinant protein was expressed in E. coli strains and purified under denaturing conditions using nickel affinity chromatography. In the end, purification of the proteins was followed by sequential dialysis refolding and quantification built on the BCA protein assay to ascertain purity and consistency.

Infection Challenge and Protection Assessment

Two weeks after their last immunisation, mice were challenged transurethrally with UPEC strain CFT073 at 10^8 CFU, leading to an ascending UTI. The bacterial colonisation was assessed 48 h post-infection by enumeration of CFUs from urine, bladder, and kidney tissues. The used tissues were homogenised, plated on an LB agar dish, and kept overnight in an incubator to quantify the bacterial burden. The efficacy of vaccinated protection was compared against the controls by determining the burden of bacteria.

Immune Response Evaluation

1. Humoral Immune Response

This study collected serum and urine samples before and after immunization to analyze the antigen-specific antibody responses. Enzyme-linked immunosorbent assays (ELISA) were performed to quantify IgM, IgG, and IgA levels. The class switch from IgM to IgG was described by a switch index used to evaluate the strength of the humoral response. This index represented the relationship between antibody production and bacterial clearance in the infected tissues.

2. Cytokine Analysis

Splenocytes were isolated from immunised mice and stimulated with purified antigens to characterise the cellular immune response. Cytokine levels of IFN-γ and IL-17 were measured in culture supernatants using the ELISA. Cytokines were selected because they mediate such protective immune responses against bacterial infections. Cytokine production pre- and post-infection was studied to identify vaccine-induced cellular immune responses.

Results

Candidate Antigen Selection

A functional vaccinology approach combining genomics and proteomics was employed to identify likely vaccine candidates for Uropathogenic *Escherichia coli* (UPEC) infections. Such strategy specifically set out some criteria for determining the ideal UPEC vaccine to be viewed in these conditions: high in vivo expression, induction undergrowth in human urine, surface exposure, antigenicity, and pathogen specificity. Out of the proteome analysis of the E. coli pyelonephritis strain CFT073, six proteins met the above conditions (Table 1). These six candidates were identified as outer membrane iron receptors: ChuA, Hma, IutA, IreA, Iha, and IroN.

It is revealed by genomic data that the iron-acquisition machinery-encoding genes were distinctive from other genes elevated the most in CFT073 during its infection of murine urinary tracts. Proteomics revealed that these iron receptors constituted among the top 15% of proteins most induced during growth in human urine. Furthermore, immunoproteomics investigations confirmed that these antigens invoked humoral immune responses during experimental UTIs. Comparative genomic studies have also provided added credence to the pathogen specificity of these proteins, revealing that genes coding for ChuA, Hma, IroN, and IutA were remarkably more common in UPEC strains than in commensal E. coli.

Table 1: Candidate Antigens and Selection Criteria

Gene	Locus Tag	Fold Change in Vivo	In Vivo Percentile	Fold Change in Human Urine	Antigenic	% UPEC (n = 60)	% Fecal (n = 35)
ChuA	c4308	7.85	94.3	25.6	+	85	28
Hma	c2482	6.92	93.1	13.4	+	72	22
Iha	c3610	18.5	88.7	5.92	+	52	39
IreA	c5174	21.8	92.4	7.21	+	30	18
IroN	c1250	19.5	84.2	6.74	+	65	29
IutA	c3623	5.92	96.8	45.2	+	68	15

Vaccination Confers Protection Against Experimental UTI

The six iron receptor antigens recognized were expressed as recombinant proteins. After purification, they were crosslinked chemically to cholera toxin (CT) to be used as an adjuvant. Female mice were grouped (n = 18–25) and given intranasal vaccinations containing either antigen-CT complexes or CT alone as a control. Post-vaccination, mice were transurethrally challenged with UPEC strain CFT073, and 48 hours later, the colonisation levels were assessed by measuring CFUs in urine, bladder, and kidney tissues.

Three of the six antigens provided a significant level of protection against UPEC challenge. Mice vaccinated with the heme receptor Hma presented a 2.5-log reduction in CFU/g with UPEC infection in the kidneys compared to controls on the 11th-day post-infection ($p = 0.01$), with no bacterial colonization detected in 12 of 20 tested mice (<100 CFU/g). For IreA, a 2-log reduction was noted in the bladders of vaccinated mice compared to controls, with 10 of 18 mice having no detectable bacteria (<100 CFU/g) in the bladder. The IutA receptor types in aerobactin showed lowered median CFU/g in both bladder and kidney tissues, with 1-log reductions at each site; for the bladder, $p = 0.03$, and for the kidney, $p = 0.02$, respectively. Neither ChuA nor Iha was protective in a significant fashion, with the latter being additionally associated with severe side effects leading to a mortality rate of 30% in vaccinated mice.

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Table 2: Bacterial Colonization in Vaccinated Mice Following UPEC Challenge

Antigen	Median CFU/g in Bladder	p-Value (Bladder)	Median CFU/g in Kidneys	p-Value (Kidneys)	Mice with Undetectable CFU (n)
ChuA	5.1×10^5	0.12	7.3×10^4	0.10	2/18
Hma	1.7×10^3	0.02	3.4×10^2	0.01	12/20
Iha	4.9×10^5	0.15	6.8×10^4	0.12	3/18
IreA	2.1×10^3	0.02	4.6×10^3	0.04	10/18
IroN	3.6×10^4	0.08	5.2×10^3	0.09	6/20
IutA	8.2×10^3	0.03	9.4×10^3	0.02	8/20

Heterologous Protection

The antigens Hma and IreA were further evaluated for their ability to provide protection against a heterologous UPEC strain, 542. Mice vaccinated with Hma showed a 1.5-log reduction in median CFU/g in the bladder ($p = 0.03$). Similarly, IreA vaccination led to a 2-log reduction in median CFU/g in the kidneys ($p = 0.04$). These results highlight the potential of these antigens to confer broad-spectrum protection against diverse UPEC strains.

Cytokine Responses

Splenocytes from vaccinated mice were stimulated with corresponding antigens to assess cellular immune responses, and the secretion of proinflammatory cytokines IFN-γ and IL-17 was measured. Splenocytes from mice immunised with protective antigens (Hma, IreA, and IutA) secreted significantly higher levels of both cytokines compared to controls ($p < 0.05$). Post-challenge, a decrease in cytokine secretion was observed, indicating an adaptive immune response modulated by vaccination. Non-protective antigens (ChuA and Iha) elicited minimal cytokine production, further corroborating their limited efficacy.

Table 3: Cytokine Responses Following Vaccination

Antigen	IFN-γ (pg/mL)	p-Value	IL-17 (pg/mL)	p-Value
CT (Control)	12.5	-	8.2	-
ChuA	15.4	0.08	10.1	0.09
Hma	58.3	0.001	45.6	0.002
Iha	18.7	0.06	12.3	0.08
IreA	51.2	0.002	39.8	0.003
IroN	22.5	0.04	19.2	0.05
IutA	47.3	0.001	38.7	0.002

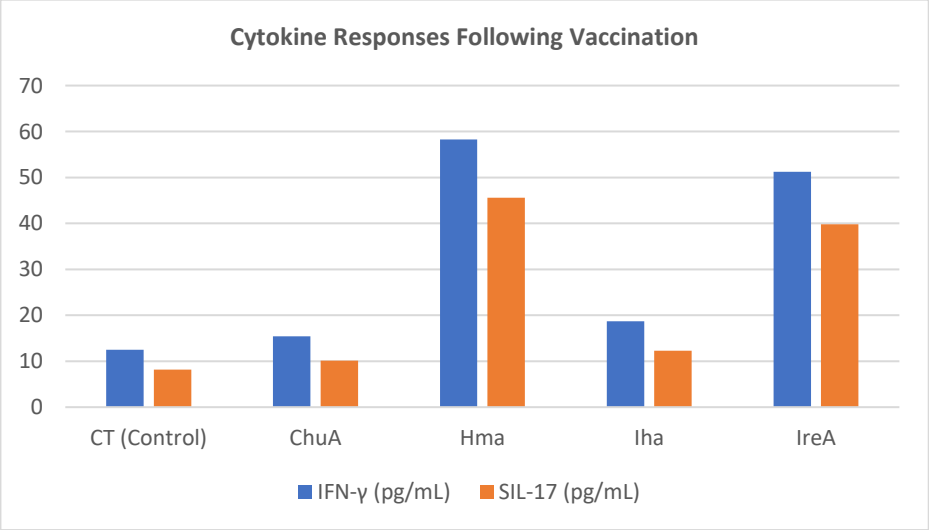

Figure 1: Cytokine Responses Following Vaccination

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Antigen-Specific IgA in Urine

To evaluate mucosal immunity, antigen-specific IgA levels were measured in urine. Vaccinated mice with Hma and IreA demonstrated the highest fold increases in IgA levels, with 30-fold and 8-fold increases, respectively, compared to controls. Elevated IgA levels were strongly correlated with reduced bladder colonization ($p = 0.02$). Non-protective antigens and peptide-based immunizations failed to elicit significant IgA responses.

Table 4: Antigen-Specific IgA Fold Change in Urine

Antigen	IgA Fold Change	p-Value
ChuA	2.1	0.09
Hma	30.2	0.02
Iha	1.8	0.11
IreA	8.3	0.02
IroN	3.6	0.08
IutA	6.7	0.04

Systemic Antibody Responses

Serum antibody responses were measured pre-and post-vaccination. Mice vaccinated with protective antigens significantly increased antigen-specific IgG and IgM levels ($p = 0.001$). A calculated Class Switch Index demonstrated a strong correlation between antibody class-switching from IgM to IgG and reduced bacterial colonization in the bladder ($p = 0.005$).

These results indicate that targeting UPEC iron acquisition systems can effectively reduce bacterial colonization and elicit robust systemic and mucosal immune responses, highlighting the potential of these antigens for UTI vaccine development.

Table 5: Systemic Antibody Responses Following Vaccination

Antigen	IgM Fold Increase	IgG Fold Increase	Class Switch Index	Correlation with Bladder CFU (p-Value)
CT (Control)	1.2	1.5	0.12	-
ChuA	1.8	2.2	0.32	0.15
Hma	5.6	10.1	1.45	0.001
Iha	1.5	2.1	0.29	0.12
IreA	4.3	8.7	1.12	0.002
IroN	2.3	3.9	0.51	0.08
IutA	4.9	9.8	1.37	0.002

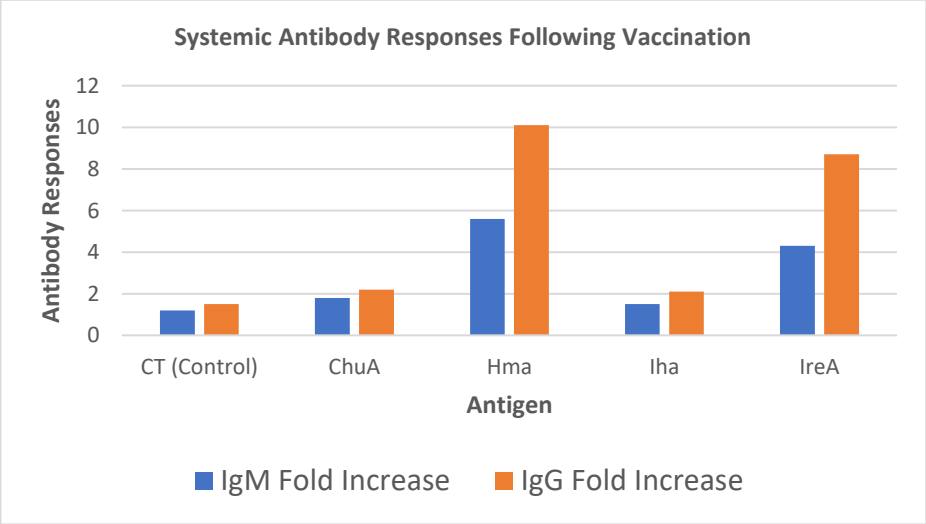

Figure 2: Systemic Antibody Responses Following Vaccination

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Discussion

The essential health concern surrounding urinary tract infections due to uropathogenic *Escherichia coli* (UPEC) is morbidity and relapse. The investigation of an effective vaccine against UPEC infection could provide significant aid in mitigating this burden. The authors centred their study around a systematic functional vaccinology approach, basically aimed at very promising vaccine candidates concerning proteins related to iron acquisition, which is most critical to UPEC pathogenesis **[15]**. Of the 5300 predicted proteins encoded by the E. coli CFT073 genome, six outer membrane iron receptors (ChuA, Hma, Iha, IreA, IroN, and IutA) were chosen for their characterization as vaccine targets based on properties such as surface localization, conservation among UPEC strains, antigenicity, and upregulation during infection and growth in human urine **[14]**.

Immunization with three of these antigens (Hma, IreA, and IutA) by the intranasal route evoked significant antigen-specific immune responses, both humoral and cell-mediated, conferred substantial protection from experimental UTI in mice **[16]**. Antigen-specific production of IL-17 and IFN-γ, with significant IgA raised in urine and IgG in serum, correlated with decreased bacterial colonization within the bladder and kidneys. These findings also highlight the worth of targeting iron acquisition pathways in designing vaccines against UTI **[17]**.

Site-Specific Protection

A wide dispersion of site-specific protection in the vaccines depends on the biological roles of antigens in UPEC colonization and pathogenesis. Mice immunized with the IreA vaccine displayed significant protection in the bladder, showing an over 2-log reduction in bacterial colonization and 60% of vaccinated mice achieving undetectable bacterial levels **[18]**. This bladder protection could be due to the induction of antigen-specific IgA in the urine, which correlates with diminished bacterial colonization. The reduced bacterial count in the kidneys of IreA-vaccinated mice suggests that protection of the bladder hinders the ascent of the bacteria to the kidneys **[19]**.

In contrast, Hma vaccination provided significant kidney-specific protection, with over 50% of immunized mice showing no detectable bacterial colonization in the kidneys. However, Hma vaccination did not reduce bladder colonization, which likely reflects the specific role of Hma in kidney colonization **[12].** The kidney-specific protection would involve antibody-mediated interference with the receptor's function or immune targeting of bacteria expressing Hma in a tissue-specific way.

Cellular and Humoral Immune Responses

Because anti-IL-17 and anti-IFNg showed significant upregulation post-vaccination with protective antigens (Hma, IreA, and IutA), both IL-17 and IFN-γ levels rose significantly. IL-17 is responsible for recruiting neutrophils, while IFN-γ is a critical component of the immune response necessary for controlling bacterial infections **[20]**. These results suggest that antigen-specific lymphocytes migrate to the sites of infection, alluding to a view that post-challenge levels of cytokines decrease significantly because of the uptake of the infections. Notably, this effect was partly prominent for the IreA vaccine, with pronounced bladder protection **[21].**

Indeed, humoral immunity provides an essential aspect of protection. IreA and IutA enhance IgA production in the urine, which is associated with decreased bladder colonisation. This mucosal immune response points to the role of IgA in protection mucosally at the site of infection **[22]**. The levels of serum IgG dramatically increased after vaccination, though more pronounced for Hma and IutA, as they were shown to give some protection to kidneys. The detected switching from IgM to IgG class was strongly associated with a decline in bacterial

colonisation, reinforcing the role of systemic antibody responses in immunity **[23]**.

Comparison to Existing UTI Vaccines

Existing UTI vaccines, such as Solco-Urovac and FimH-based vaccines, have shown limited efficacy and short-term protection. Solco-Urovac generates urinary IgA but requires frequent administration to extend the protection duration. At the same time, FimH-based vaccines could protect animal models but still need to be translated into widely available clinical applications **[24]**. Our results build on this work by demonstrating that targeting the pathways of iron acquisition would rather be effective since this is an important process concerning the survival and virulence of the bacterium. In intranasal immunization, the iron receptor antigens (Hma, IreA, and IutA) induced much stronger protection than in earlier studies.

Implications for Vaccine Development

This study advocates targeting a whole class of functionally related proteins that can be called outer membrane-iron receptors instead of focusing on individual antigens. The identified antigens are found to be strongly conserved among UPEC strains while being largely undeveloped in commensal E. coli, which decreases the possibility for off-target effects to occur. Intranasal immunization, which takes advantage of immune cell migration from one mucosal site to another, provides a rational strategy for inducing remote mucosal immunity into the genitourinary tract.

As such, the protective antigens identified in this study (Hma, IreA, and IutA) may be ideal candidates for constructing multivalent subunit vaccines, thus providing broad-spectrum protection against diverse strains of UPEC in several compartments of the urinary tract. Future studies must aim to optimize the formulations of vaccine compositions that include the application of adjuvants for potent immune responses and investigations into the longevity of the immune protective response.

Conclusion

The study suggests that targeting UPEC iron acquisition systems for UTI vaccine development will mitigate recurrence, time lost and economic impact. Protective antigens Hma, IreA, and IutA induce strong mucosal and systemic immune responses, significantly reducing bacterial colonization in the bladder and kidneys. Future directions of research should be devoted to vaccine formulation optimization and the study of their long-term efficacy.

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