Novel vaccine antigen combinations elicit protective immune responses against *E. coli* sepsis

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ABSTRACT

Systemic infections caused by extraintestinal pathogenic *E. coli* (ExPEC) have emerged as the most common community-onset bacterial infections and are major causes of nosocomial infections worldwide. The management of ExPEC infections has been complicated by the heterogeneity of ExPEC strains and the emergence of antibiotic resistance, thus their prevention through vaccination would be beneficial. The protective efficacy of four common ExPEC antigen candidates composed of common pilus antigens EcpA and EcpD and iron uptake proteins IutA and IroN, were tested by both active and passive immunization in lethal and non-lethal murine models of sepsis. Additionally, antibody raised to a synthetic form of a conserved surface polysaccharide, β -(1-6)-linked poly-*N*-acetylglucosamine (dPNAG) containing 9 monomers of (non-acetylated) glucosamine (9GlcNH₂) conjugated to tetanus toxoid TT (9GlcNH₂-TT) was tested in passive immunization protocols. Active immunization of mice with recombinant antigens EcpA, EcpD, IutA or IroN elicited high levels of total IgG antibody of IgG1/IgG2a isotypes, and were determined to be highly protective against *E. coli* infection in lethal and non-lethal sepsis challenges. Moreover, passive immunization against these four antigens resulted in significant reductions of bacteria in internal organs and blood of the mice, especially when the challenge strain was grown in iron-restricted media. Inclusion of antibodies to PNAG increased the efficacy of the passive immunization under conditions where the challenge bacteria were grown in LB medium but not in iron-restricted media. The information and data presented are a first step towards the development of a broadly protective vaccine against sepsis-causing *E. coli* strains.

Key words: vaccine; sepsis; antibodies; sepsis; antigens; challenge

1. Introduction

Extraintestinal pathogenic *E. coli* (ExPEC) normally reside in the human intestine but are capable of infecting extraintestinal sites like the blood, urinary tract, and meninges, using specific virulence attributes [1, 2]. ExPEC are major causes of both community and nosocomial bacterial sepsis, with mortality ranging from 30-50% [3-5]. Clinical failure of antibiotic therapies, mainly due to multidrug resistance, increases the cost of care and results in prolonged morbidity for patients [6]. As a result, the prevention and control of these infections is a pressing concern.

A protective vaccine would be a useful strategy to prevent ExPEC infections. Efforts to develop vaccines against ExPEC have previously focused on specific virulence factors (O-antigens, OMP fractions, fimbriae, toxins, and iron-acquisition systems), or whole cells, but most of them were either not safe, poorly immunogenic, or did not provide cross-protection against ExPEC strains [7-12].

In order to develop a more effective vaccine against ExPEC sepsis, we tested siderophore receptors (IutA and IroN), which are highly prevalent among human ExPEC isolates [13]; and *E. coli* common pilus (ECP) [14] that plays a synergistic role in multiple steps of the infectious process [15-18]. Additionally chosen for passive vaccine studies were antibodies raised to a synthetic, deacetylated glycoform of the bacterial surface polysaccharide poly- β -(1-6)-*N*-acetylglucosamine (PNAG), a 9-mer of β -(1 $\rightarrow$ 6)-d-glucosamine conjugated to a tetanus-toxoid carrier

protein (9GlcNH₂-TT), previously identified as a potential universal vaccine against pathogenic bacteria, including *E. coli* [19].

2. Methods

2.1. Ethics Statement

New-Zealand-White rabbits and female BALB/c mice were obtained from Charles River Labs (Wilmington, MA). Vaccination and infection of animals were performed in accordance with protocols approved by the Arizona State University (ASU) Institutional Animal Care and Use Committee (IACUC) in dedicated facilities at the Biodesign Institute, ASU (Protocol number 1168R).

2.2. Antigens preparation

Genes encoding the selected antigens (EcpA, EcpD, IutA, IroN) (**Table S1**) were PCR amplified and cloned into pET-101/D-TOPO® vectors (Invitrogen). Recombinant proteins were expressed in *E. coli* BL21 and purified from inclusion bodies as His-tagged protein, using ProBond Ni-NTA resin columns (Invitrogen). The expressed proteins were 78 kDa (IroN), 74 kDa (IutA), 45 kDa (EcpD), and 21 kDa (EcpA), respectively.

2.3. Production of rabbit antibodies

Antisera to EcpA, EcpD, IutA, and IroN were raised by injecting subcutaneously (s.c.) rabbits with 250 µg of individual recombinant antigens (rAgs) in complete Freund's adjuvant, followed by two boosts at 3 weekly intervals with 250 µg of rAg in incomplete Freund's and two boosts in Montanide™ ISA 71 VG adjuvant. The concentration of antigen-specific rabbit IgG was measured by indirect ELISA using a goat-derived anti-rabbit IgG standard (Southern Biotech, Birmingham, AL). Rabbit antibodies raised to 9GlcNH₂-TT were prepared as previously described [19].

2.4. Bacterial challenge strain

Mice were challenged with urosepsis *E. coli* CFT073 [20] (**Table S1**) grown in either Lysogeny Broth (LB) [21] at 37°C with or without 2,2'-bipyridyl (100 µM) with aeration until an OD₆₀₀ of ~0.85 or in Dulbecco's Modified Eagle Medium (DMEM) + 0.5% Mannose + 2,2'-bipyridyl (100 µM), at 28°C for 48h standing and the OD₆₀₀ value of culture was adjusted to ~0.85. The strain was stored at -80°C in peptone-glycerol medium.

According to NCBI's BLASTn, the genome of CFT073 (AE014075.1) contains the sequences for *ecpA(matB)* (NP_752341.1), *ecpD(yagW)* (NP_7523338.1), *iutA* (NP_755498.1), *iroN* (AAN79707.1), and *pgaABCD* locus encoding [22].

2.5. Vaccination and challenge

2.5.1. Active immunization

As shown in Figure S1, mice were s.c. injected with rAgs, either alone or in combinations [two or four antigens (high or low doses)] (**Table S2**), in phosphate buffered saline (PBS), emulsified in ISA 71 VG adjuvant, and boosted on day 23 (**Table S2**). On days 21 and 40, blood was collected by submandibular bleeding and sera were stored at -80°C. On day 42, mice were challenged intraperitoneally (i.p.) with CFT073. In the non-lethal challenge ($\sim 3.5 \times 10^7$ CFU), necropsies were performed after 24 or 48 h of challenge. In the lethal challenge ($\sim 1.8 \times 10^8$ CFU), death was recorded for 48 hours after inoculation; survived mice were euthanized and necropsied. Bacterial loads were determined in blood and organs.

2.5.2. *Passive immunization*

Mice were i.p. immunized with 200 μ l of either pre-immune rabbit serum (control) or antigen-specific rabbit IgG antiserum (50 μ g). Twenty-hour later, mice were boosted with the same serum samples. After a further four-hour, mice were i.p. challenged with $\sim 1.8 \times 10^8$ CFU of CFT073 grown under different conditions as described above. Necropsies were performed 24 hours post-challenge with enumeration of bacteria in blood and organs.

2.5.3. *Serum antibody titers*

Antigen-specific IgG and IgG1/IgG2a isotypes titers in serum against each antigen were determined by ELISA [23], in 96-well plates, using a rAg coating concentration of 2 μ g/ml. The endpoint titer was defined as the reciprocal of the highest dilution that gave an OD₄₀₅ twice that of the control.

2.6. Statistical analysis

Comparison analyses of data were performed using Kruskal–Wallis test followed by Dunn's multiple comparisons (bacterial clearance and ELISA data) and Fisher's exact and the Log-rank (Mantel-Cox) tests (Survival and mean death time data). $P < 0.05$ were considered statistically significant. All statistical tests were performed using GraphPad Prism 6.0 software.

3. Results

3.1 Expression of selected antigens

As determined by immunoblotting [24], CFT073 expressed PNAG in all conditions tested; when grown in LB at 37°C [25], it expressed IutA and IroN at low levels, but not Ecp. In iron-restricted LB, a condition known to induce over-expression of iron-regulated outer-membrane proteins (IROMPs) [26], CFT073 over-expressed IutA and IroN, but did not express Ecp. Finally, in iron-restricted DMEM at 28°C, it expressed Ecp and iron-uptake antigens at high levels.

3.2. Antigen-specific IgG elicited in vaccinated mice

Evaluation of antigen-specific (EcpA, EcpD, IutA, and IroN) total IgG in pooled-sera from 3 mice (equal volumes) in each group at days 21 and 40 post-immunization determined that IgG responses against the antigens varied depending on the vaccine composition (**Fig. 1A**). While

anti-EcpA IgG were detected as early as day 21 post-vaccination in all EcpA-vaccine groups and persisted or even increased at day 40 post-vaccination, anti-IutA and anti-IroN IgG were only detected at day 40 post-vaccination in all IutA- and IroN-groups. In the EcpD groups, with the exception of the group vaccinated with all antigens at a low dose, in which anti-EcpD IgG were detected only at 40 day post-vaccinations, anti-EcpD IgG were detected at day 21 and persisted and even increased at day 40 post-vaccination in other groups (**Fig. 1A**). Our data have also shown that antibodies against specific antigens are cross-reactive with other antigens tested (**Fig. 1**).

3.3. Level of IgG titers elicited in mice

In a repeat experiment with a similar design, but in which serum samples tested were collected at day 40 and were not pooled, we determined that the levels of IgG elicited in different groups varied (**Fig. 1B**).

In the EcpA-containing groups, EcpA elicited significantly ($P<0.0017$) lower IgG titers compared to those elicited in EcpA+EcpD or all antigens at high dose groups. However, EcpA+EcpD and all antigens at both low and high dose, elicited a similarly high level of anti-EcpA IgG in mice.

In the EcpD-containing groups, although the differences were not significant, all antigens at a high dose elicited the highest level of anti-EcpD IgG compared to all other groups.

184 In the IutA-containing groups, the anti-IutA IgG elicited in IutA+IroN or all antigens at a low
185 dose groups were similar levels and were significantly lower than that induced by all antigens at
186 a high dose ($P=0.0144$ and $P=0.0199$, respectively).

187
188 In the IroN-containing groups, the levels of anti-IroN IgG in IroN, IutA+IroN, or all antigens
189 at a low dose groups were lower than the one in all antigens at a high dose, with the differences
190 being significant with IutA ($P=0.0037$) and IutA+IroN ($P=0.0151$) groups.

191 192 *3.4. IgG1 and IgG2a responses in mice*

193
194 Our data showed that all four antigens tested, either alone or in combinations, induced both
195 IgG1 (**Fig. S2A**) and IgG2a (**Fig. S2B**) isotypes in vaccinated mice at day 40 post first
196 vaccination.

197 198 *3.5. Vaccine efficacy*

199
200 Mouse models of non-lethal and lethal sepsis were used to assess the protective efficacy of
201 the vaccines against the challenge with CFT073 grown either in LB (**Fig. S3**) or iron-restricted
202 DMEM (**Fig. 2**). In the non-lethal challenge, mice vaccinated with all antigens at the higher dose
203 had lower bacterial loads at 48h post-challenge compared to the non-vaccinated mice, with the
204 differences being significant in liver ($P=0.0349$) (**Fig. S3**). Two mice died before necropsy, one
205 in each of the groups vaccinated with EcpA and all antigens at high dose, respectively.

The percentage of mice (mice died before necropsy not-included) without *E. coli* in the blood was higher in the groups vaccinated with all antigens (78%); EcpA (67%); IutA, EcpA+EcpD, or IutA+IroN (all 40%) than the PBS group (30%), and lower in EcpD (10%) and IroN (20%) groups (**Fig. S3**).

In the second experiment of non-lethal sepsis, at 24h post-challenge with CFT073 grown in iron-restricted DMEM, mice immunized with all antigens at a high dose had fewer bacteria in organs and blood compared to the non-vaccinated group, with the difference being significant in spleen ($P=0.0011$) (**Fig. 2**).

In the model in which mice were challenged with a lethal dose ($\sim 1.8 \times 10^8$ CFU) of CFT073 grown in iron-restricted DMEM, all vaccinated groups had better survival than the PBS control group. The differences in percentage survival (PS) and/or mean death times (MDT) were statistically significant for some groups, including EcpA (MDT, $P=0.024$); EcpD (PS, $P=0.046$; MDT, $P=0.017$); EcpA+EcpD (PS, $P=0.0003$; MDT, $P=0.0001$), and all antigens at the highest dose (PS, $P=0.0013$; MDT, $P<0.0001$) and low dose (MDT, $P=0.0103$). Mice immunized with the EcpA+EcpD, all antigens at high dose and the EcpD had the highest rate of survivals (75%, 67%, and 55%, respectively), followed by the EcpA, IutA+IroN, and all antigens at a low dose groups, which had 33% survival; and finally, the IroN or IutA groups had the lowest survivals (27% and 25%, respectively) (**Fig.3A, Table 1**). Among the mice that survived, although differences were not significant, those vaccinated with EcpA+EcpD or all antigens at a high dose had, in general, lower bacterial loads in spleens, livers, and blood than mice vaccinated with individual antigens (**Fig.3B**).

230
231 3.6. Protective efficacy of antisera raised to EcpA, EcpD, IutA, IroN or 9GlcNH₂-TT against
232 CFT073 infection
233

234 We previously determined that anti-9GlcNH₂-TT serum protected mice against lethality
235 following i.p. challenge with PNAG-positive urinary tract infection (UTI) *E. coli* isolates [19].
236 Here we evaluated the efficacy of anti-9GlcNH₂-TT serum alone (**Fig. 4**) or in combination with
237 anti-(EcpA, EcpD, IutA, and IroN) sera (**Fig. 5**) to CFT073 in a non-lethal sepsis model. As
238 shown in **Fig. 4**, mice vaccinated with the anti-9GlcNH₂-TT serum had lower bacterial loads in
239 blood and organs than those of mice vaccinated with pre-immune serum, with the differences
240 being significant in spleen ($P=0.0171$) and blood ($P=0.0150$).
241

242 In a second experiment, we vaccinated mice with anti-(EcpA, EcpD, IutA, and IroN) sera
243 with or without anti-9GlcNH₂-TT serum, and i.p. challenged with CFT073 grown in different
244 conditions (**Fig. 5ABC**). Based on the significance of the differences in bacterial loads in organs
245 and blood comparing immune-serum-vaccinated mice with pre-immune serum vaccinated mice,
246 those vaccinated with anti-(EcpA, EcpD, IutA and IroN) sera had lower burdens of bacteria
247 grown in iron-restricted media (LB and DMEM) (**Fig. 5BC**) than those grown in LB (**Fig. 5A**).
248 Addition of anti-9GlcNH₂-TT serum to the vaccines improved the mice's ability to control
249 bacterial tissue levels after challenge with bacteria grown in LB (**Fig. 5A**).
250

251 **4. Discussion**
252

Although there are currently no human vaccines available to prevent ExPEC infections, much progress has been made in recent years in identifying potential ExPEC antigens and their evaluation for protection in mice [7-12, 27]. Our strategy to design an efficacious vaccine is to combine protein antigens involved in different steps of ExPEC infections to increase the likelihood of preventing these bacteria from causing disease. We thus tested vaccines that contain antigens for adhesins (EcpA, EcpD) and iron uptake (IutA, IroN), for their ability to elicit protective immune response in sepsis mouse models.

IROMPs have been suggested as good candidates for vaccine components against some Gram-negative bacterial infections [8, 25, 28, 29]. Additionally, since salmochelin and the hydroxamate aerobactin are considered key virulence factors of these bacteria [30], we included their receptor proteins (IroN, IutA) into our vaccine. We also selected the adhesin antigens of ECP (EcpA and EcpD) [31]. This antigen, common among *E. coli*, is involved in the early-stage of bacterial biofilm formation and host cell recognition [14]. Prevention of these functions by targeting ECP would thus help prevent the outcome of these diseases.

Detection of antibodies against the specific antigens in the vaccinated mice was not surprising, as the ability of EcpA [14], EcpD [31], IutA [32], and IroN [8] to elicit antibodies in mice and/or rabbits was previously reported. However, we showed that antibodies to these antigens are elicited at different time periods following immunization in mice. The ability of Ecp antigens, but not IutA and IroN to elicit IgG antibodies as early as two weeks post-vaccination, as well the need for a boost in the EcpD group vaccinated with all antigens at low dose to elicit anti-EcpD IgG, indicate that both the nature of the antigens or their concentrations in a vaccine

affected the elicitation of the immune response; more studies are needed to elucidate these phenomena. The cross immunity between antigens EcpA, EcpD, IutA, and IroN could be due to shared epitopes, more analyses on these antigens are needed to confirm this hypothesis.

We also showed that the level of IgG elicited in the serum of vaccinated mice was affected by the nature, combinations, and dose of antigens injected. Among all vaccine formulas tested, immunization with quadrivalent vaccine at a high dose elicited the highest level of IgG. The inability of the vaccine at low dose and with individual antigens, which both had the same total concentration of proteins (20 µg), indicates that the combination of the four antigens as well the concentration of each antigen in the formula are important to elicit the highest level of IgG.

Since UTIs are the most common site of *E. coli* sepsis and urosepsis *E. coli* are highly virulent [33-36]; we tested the protective abilities of our vaccines against the highly virulent urosepsis *E. coli* CFT073 in mouse sepsis models. In general, vaccinated mice were better protected than the non-vaccinated mice, as determined by the decrease in the bacterial loads in the blood and internal organs in non-lethal challenge and better survivability in lethal challenge of vaccinated mice. Among all vaccine formulas tested, EcpA+EcpD, all four antigens at a high dose, and EcpD, conferred high protection. The ability of EcpA+EcpD to elicit superior protection (75%) than IutA+IroN (33%) indicates the importance of including multifunctional virulence factors in an ExPEC vaccine. However, although EcpA+EcpD had slightly higher protection ability than the quadrivalent vaccine (67%), this later would provide broader protection because the inclusion of both Ecp and iron-acquisition antigens in the vaccine.

Compared to previous studies that tested nine conserved ExPEC antigens [27] and a multi-epitope subunit vaccine that included IutA and IroN epitopes in a similar mouse sepsis model [25], the use of a higher bacterial challenge dose (10X and 100X respectively) in our studies, indicate possible protective superiority of our vaccine.

PNAG, a conserved surface polysaccharide produced by major bacterial pathogens, including *E. coli* [37], is required for optimal fitness of ExPEC during both UTI and systemic infections [38]. We previously demonstrated the protective efficacy of anti-9GlcNH₂-TT serum against PNAG-positive UTI *E. coli* isolates [19] in a sepsis mouse model. Herein, using the same model, we showed the protective efficacy of the anti-9GlcNH₂-TT serum against CFT073 sepsis challenge. The inability of anti-9GlcNH₂-TT serum to protect against PNAG-negative *E. coli* isolates [19] indicates that its combination with other antigens could be better and more broadly protective against ExPEC isolates, as some do not synthesize PNAG.

In order to mimic the *in vivo* conditions encountered by the bacterium, the challenge strain CFT073 was grown in different conditions. The superior protection of anti-(EcpA, EcpD, IutA, and IroN) sera against the challenge strain grown in iron-restricted DMEM; could be explained by the fact that the challenge strain expressed all antigens in this condition and iron-uptake are over-expressed in iron-limited media. The ability of anti-9GlcNH₂-TT serum included with the other four antigen-specific sera to greatly increase the efficacy of the vaccine when challenged with the strain grown in LB, is due to the fact that PNAG is expressed in both LB and iron-restricted media. Future studies using other mouse models of human ExPEC infections, such as

transurethral challenge [39], could determine if our vaccine would have protection against other ExPEC infections, including UTI, as previously tested with the IutA and IroN antigens [8].

Our vaccine will diminish, if not ultimately eliminate, pathogenic *E. coli* with minimal cross-reactivity with the healthy flora, because IroN and IutA antigens targeted within this vaccine are more prevalent among ExPEC than commensal *E. coli* [40-42]. Regarding the ECP antigen, since it has been shown that in *in vitro* conditions mimicking the *in vivo* situation, only virulent *E. coli* highly produced ECP, whereas commensal *E. coli* did not [43]; as our vaccine will only target ECP-expressing *E. coli in vivo*, it would not have effect on non-ECP-expressing commensal *E. coli*. Although further studies to confirm the safety of the vaccine are needed, in this study, rabbits immunized with high doses of recombinant IutA, IroN, EcpA, and EcpD for antibody production thrived for 12-36 months under frequent veterinary care and observation without displaying any noticeable signs of distress, illness or weight loss. Moreover, Phase-I evaluation of MAb-F598 (anti-PNAG antibodies) in humans revealed no signs or symptoms associated with disruption of normal flora.

5.CONCLUSION

The significant increases in humoral immunity and decreases in CFU recovery in mice immunized with antigens EcpA, EcpD, IutA, and IroN depict a first step towards the development of a broadly protective vaccine against sepsis *E. coli*. Treatment with antibodies against the four antigens protected against sepsis ExPEC infection and inclusion of serum anti-9GlcNH₂-TT improved the protective effect and could lead to broader protection.

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Table 1. Survival rates and mean death times of mice vaccinated with either PBS (control) or different recombinant antigens individually or in combination.

Vaccine Groups	Survival rates/mean death times			
	48hr. Survival		Mean Death Time	
	N _{alive} /N _{total} (%)	P value	(Hours) ± SD	P value
PBS	0/12 (0%)		22.2 ± 9.2	
EcpA	4/12 (33%)	NS	24.8 ± 10.5	<i>P=0.024</i>
EcpD	6/11 (55%)	<i>P=0.046</i>	28.5 ± 15.3	<i>P=0.017</i>
IutA	3/12 (25%)	NS	20.4 ± 8.0	NS
IroN	3/11 (27%)	NS	21.0 ± 8.5	NS
EcpA+EcpD	9/12 (75%)	<i>P=0.0003</i>	23.8 ± 16.3	<i>P=0.0001</i>
IutA+IroN	4/12 (33%)	NS	18.0 ± 2.5	NS
EcpA+EcpD+IutA+IroN (high dose)	8/12 (67%)	<i>P=0.0013</i>	34.2 ± 13.1	<i>P<0.0001</i>
EcpA+EcpD+IutA+IroN (low dose)	4/12 (33%)	NS	28.0 ± 11.4	<i>P=0.0103</i>

N_{alive}/ N_{total}, number of mice alive of total mice tested; significant *P* values compared to PBS group compared to PBS using Fisher's exact test (survival), and Log-rank (Mantel-Cox) test (mean death times); NS, not significant.

Figure legends

Figure 1. Total IgG responses against specific antigens. (A) Data represents IgG antibody levels induced in BALB/c mice immunized with either PBS or antigens (IutA, IroN, EcpA, and EcpD) either individually or in combinations at day 21 and 40 post vaccination. From each experiment, the sera from 3 mice of each group were pooled and tested in duplicate. (B) bars and error bars represent the mean and SD values from 10 individually tested mice from each group. Mice were immunized with either PBS or antigens (IutA, IroN, EcpA, and EcpD) either individually or in combinations, and analyzed individually by IgG ELISA against specific antigens. The values are shown as $\log_{10}$ IgG total titer. Statistically significant differences were determined by a Kruskal–Wallis test followed by Dunn's multiple comparison ($P < 0.05$).

Figure 2. Effect of vaccination on bacterial load in organs (spleen, liver) and blood at 24 hours post-challenge. Mice were vaccinated with either PBS (control) or with all rAgs combined (high dose) and challenged with $\sim 5.5 \times 10^7$ CFU of CFT073/mouse grown in DMEM + 0.5% mannose + 2,2'-bipyridyl standing for 48 h at 28°C. Data are expressed as log CFU/g or ml found at time of necropsy (24 h post-challenge). Significant P values in comparison to the PBS group were determined by two-way ANOVA ($P < 0.05$).

Figure 3. Protective ability of rAgs against intraperitoneal challenge with CFT073. (A) Data for survival and (B) bacterial loads in liver, spleen, and blood of surviving mice are presented. Forty-two days after the first vaccine injections, BALB/c mice (n=10/group) were challenged IP with strain CFT073 grown in DMEM + 0.5% mannose + 2,2'-bipyridyl standing for 48 h at

28°C then challenged with either $\sim 2 \times 10^8$ CFU/mouse. Mice were regularly monitored for being moribund or dead for up to 48 h after infection. Surviving mice in **(B)** were euthanized, necropsied, and the bacterial loads (Log) in organs and blood were assessed. Mice who died had bacterial loads very high ($> 10^8$ CFU) in their organs (data not shown). Data are representative of 2 experiments of similar design. No statistically significant differences were determined by a Kruskal–Wallis test followed by Dunn's multiple comparison ($P < 0.05$).

Figure 4. Passive immunization with rabbit antibody to 9GlcNH₂-TT. Serum from either pre-immune rabbits or from rabbits immunized with 9GlcNH₂-TT was injected into mice (n=5 per group) who were then challenged with $\sim 2 \times 10^8$ CFU/animal of *E. coli* strain CFT073 grown in LB shaking until OD₆₀₀ ~ 0.85 . Log CFU per gram of organs (spleen and liver) or per ml of blood are presented. *P* values determined by non-parametric T-tests comparing the pre-immune and immune groups.

Figure 5. Passive immunization with rabbit antibodies to multiple antigens and challenge with CFT073 grown in multiple conditions. Groups of 10 BALB/c mice were immunized with antigen-specific sera to EcpA, EcpD, IutA, and IroN with and without anti-9GlcNH₂-TT serum and challenged with $\sim 2 \times 10^8$ CFU of CFT073 grown in: **(A)** LB shaking at 37°C; **(B)** iron-restricted LB shaking at 37°C or iron-restricted-DMEM standing at 28°C for 48 h. Log CFU per gram of organs (spleen and liver) or per ml of blood are presented. Statistically significant differences were determined by a Kruskal–Wallis test followed by Dunn's multiple comparison ($P < 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5