Development and Qualification of an Opsonophagocytic Killing Assay To Assess Immunogenicity of a Bioconjugated Escherichia coli Vaccine

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ABSTRACT The global burden of disease caused by extraintestinal pathogenic Escherichia coli (ExPEC) is increasing as the prevalence of multidrug-resistant strains rises. A multivalent ExPEC O-antigen bioconjugate vaccine could have a substantial impact in preventing bacteremia and urinary tract infections. Development of an ExPEC vaccine requires a readout to assess the functionality of antibodies. We developed an opsonophagocytic killing assay (OPA) for four ExPEC serotypes (serotypes O1A, O2, O6A, and O25B) based on methods established for pneumococcal conjugate vaccines. The performance of the assay was assessed with human serum by computing the precision, linearity, trueness, total error, working range, and specificity. Serotypes O1A and O6A met the acceptance criteria for precision (coefficient of variation for repeatability and intermediate precision, ≤50%), linearity (90% confidence interval of the slope of each strain, 0.80, 1.25), trueness (relative bias range, −30% to 30%), and total error (total error range, −65% to 183%) at five serum concentrations and serotypes O2 and O25B met the acceptance criteria at four concentrations (the lowest concentration for serotypes O2 and O25B did not meet the system suitability test of maximum killing of ≥85% of E. coli cells). All serotypes met the acceptance criteria for specificity (opsonization index value reductions of ≤20% for heterologous serum preadsorption and ≥70% for homologous serum preadsorption). The assay working range was defined on the basis of the lowest and highest concentrations at which the assay jointy fulfilled the target acceptance criteria for linearity, precision, and accuracy. An OPA suitable for multiple E. coli serotypes has been developed, qualified, and used to assess the immunogenicity of a 4-valent E. coli bioconjugate vaccine (ExPEC4V) administered to humans.

KEYWORDS assay qualification, bacteremia, conjugate vaccine, Escherichia coli, ExPEC, invasive disease, opsonophagocytic killing assay

Bacteremia and septicemia, forms of invasive disease resulting from localized infections, are caused by various bacteria and bacterial toxins in the bloodstream (1) and are a significant cause of clinical morbidity and mortality. Septicemia is the 6th most frequent cause of hospitalization (1) and is the 10th leading cause of death in the United States (2). Associated economic costs are significant, and septicemia is the most expensive condition treated in U.S. hospitals, with 2009 aggregate expenditures approaching $15.4 billion, or approximately 4.3% of all hospital costs (1, 3). Extrainestinal pathogenic Escherichia coli (ExPEC) is the organism most frequently associated with sepsis in patients with a principal sepsis diagnosis (1), with E. coli septicemia case fatality rates ranging from 5% to 30% (4). Furthermore, ExPEC strains are the most
frequent cause of urinary tract infections (UTIs) and are implicated in other localized infections, including neonatal meningitis, intra-abdominal infections, surgical site infections, osteomyelitis, soft tissue infections, pneumonia, and infections of intravascular devices (5). Increasing rates of drug resistance among ExPEC strains have led to rising rates of hospitalization, increased numbers of treatment failures, and increased treatment costs (1, 3). The rapid expansion of the multidrug-resistant O25 sequence type 131 (ST131) clone has been a significant factor in the worldwide increase in the incidence of antimicrobial-resistant ExPEC strains (6). The prevention of infection with an effective vaccine targeting the most commonly circulating ExPEC serotypes, including highly resistant strains, such as ST131 strains, could have a substantial impact in reducing the incidence of ExPEC disease, including drug-resistant infections (7).

Although there are more than 180 O serotypes of *E. coli*, most cases of human ExPEC disease are caused by between 10 and 12 O serotypes (8). Bioconjugation is a novel technology whereby polysaccharide and carrier protein are biosynthesized within *E. coli* cells, with in vivo coupling being achieved using an oligosaccharyltransferase (9). Bioconjugation can result in the production of multiple specific O polysaccharides with a homogeneous structure conjugated to any protein carrier and removes the requirement for chemical detoxification. A phase 1 study of a 4-valent *E. coli* bioconjugate vaccine (ExPEC4V) conducted in women with recurrent UTI was recently completed (10). ExPEC4V is formulated with O antigens from *E. coli* serotypes O1A, O2, O6A, and O25B coupled to a detoxified variant of the protein exotoxin A from *Pseudomonas aeruginosa* (EPA).

Development of a new vaccine requires measurement of its immunogenicity, ideally against a known correlate of protection. While the measurement of antibody levels in serum using enzyme-linked immunosorbent assay (ELISA) methods can demonstrate the immunogenic potential of a vaccine, antibody levels do not always directly correlate with protection (11). This is the case for pneumococcal conjugate vaccines (PCVs), where measurement of functional antibody responses in opsonophagocytic killing assays (OPA) may correlate with clinical protection more closely than ELISA antibody levels (12–14). OPAs measure the ability of antibodies to mediate phagocytic killing, usually in the presence of complement. Immunity to *E. coli* is via complement-mediated lysis and opsonophagocytic killing (15, 16). Cross et al. used an OPA with human neutrophils to assess the functional activity of antibodies raised by vaccination with a polyvalent O-antigen conjugate *E. coli* vaccine (17). Because the development of a multivalent ExPEC conjugate vaccine shares some of the same challenges addressed during the development of PCVs, we built on the large experience with OPAs for assessment of PCVs to establish an assay to measure functional antibody responses to an ExPEC bioconjugate vaccine (18). Here we describe the development and qualification of four singleplex OPAs for *E. coli* serotypes O1A, O2, O6A, and O25B using HL60 cells as effector cells. The OPAs described in this report are based on those described in previous studies (14, 19) with some additional modifications, such as the use of human complement rather than baby rabbit complement (BRC) and the addition of a complement preadsorption step to decrease the high levels of nonspecific killing in human serum samples.

RESULTS

**OPA optimization.** In order to adapt the previously described pneumococcal OPA protocols for use with *E. coli* strains (14, 19), the following modification were introduced into the assay: (i) an increased ratio of HL60 cells to *E. coli* cells of at least 600:1 was found to be optimal for assay performance (data not shown), (ii) human complement instead of baby rabbit complement was used, (iii) the percentage of human complement used was changed and a preadsorption step was added, (iv) fetal bovine serum was removed from the opsonization buffer (Hanks’ balanced salt solution [HBSS] with calcium, magnesium, and 0.1% gelatin comprised the assay buffer), and (v) the composition of agar used for bacterial enumeration was changed.
Initially, the *E. coli* OPA was performed using BRC. However, BRC was unable to discriminate a postvaccination response from the prevaccination condition in rat serum (Table 1). The use of human complement produced lower opsonization index (OI) titers with serum from healthy nonvaccinated subjects than the use of BRC. Only human complement yielded an increased OI titer for postvaccination serum compared with that for prevaccination serum from rats.

Additional improvement of the OPA involved adjustment of the human complement concentration and the preadsorption of complement with bacteria. A pool of human complement from three donors at 4% or 7% yielded high levels of nonspecific killing (NSK) (Table 2). Preadsorption with glutaraldehyde-treated *E. coli* at a ratio of 2 × 10^9 CFU of each strain (strains OC24452, OC24453, OC24454, and OC24176) per 1 ml of complement reduced NSK levels to <30% for a complement concentration of 4%. NSK levels where higher for strains OC24454 (76%) and OC24176 (35%) for a complement concentration of 7%. Increasing the amount of the OC24454 and OC24176 strains used in the preadsorption step to 7 × 10^9 CFU and 4 × 10^9 CFU, respectively, decreased the rate of NSK for these two strains to <42%.

In the pneumococcal OPA (20), enumeration of the bacterial colonies is done by plating the samples onto Todd-Hewitt yeast extract agar followed by placement of an overlay of Todd-Hewitt yeast extract agar containing 2,3,5-triphenyltetrazolium chloride (TTC) and incubation overnight at 37°C. These procedures resulted in the growth of *E. coli* colonies on top of as well as within the overlay, making counting of the colonies difficult. The switch to glycerol yeast extract agar (GYA) medium without an agar overlay and with a decreased incubation temperature (33°C) led to the formation of discrete colonies that could be accurately enumerated (data not shown).

To evaluate whether *E. coli* killing was mediated by an opsonophagocytic process or by the direct lytic activity of complement, OPA was performed with the removal of each of the key assay components. The results showed that in the absence of phagocytes (HL60 cells), bacterial killing was abrogated (Fig. 1). Similarly, if the complement source and serum antibodies were absent, bacterial killing was also reduced. Importantly, no direct complement lytic activity was observed when *E. coli* was incubated only with complement (Fig. 1). A statistically significant increase in the area under the curve (AUC) under all four conditions (in which key components of the assay were omitted) compared to that under the reference condition (with serum, bacteria, complement, and HL60 cells) was observed (P < 0.0001 under all four

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**TABLE 1** Comparison of baby rabbit complement to human complement using *E. coli* OC24452 (O1A)^h

| Serum sample | OI^a with: | OI^a with: | OI^a with: | OI^a with: |
|--------------|------------|------------|------------|------------|
|              | 2% BRC^b   | 5% BRC     | 10% BRC    | 2% HC^c    |
| HS^d (Sigma) | 275        | 440        | 807        | 49         |
| HS37^e       | 261        | 221        | 273        | 40         |
| HS38^f       | 30         | 124        | 116        | 19         |
| HS39^f       | 34         | 143        | 270        | 33         |
| HS40^f       | 0          | 29         | 39         | 0          |
| Rat pre-278^g| 0          | 0          | 0          | 0          |
| Rat post-278^g| 0        | 0          | 0          | 96         |
| Rat pre-378^h| 0          | 0          | 0          | 0          |
| Rat post-378^h| 0        | 0          | 0          | 90         |

^aOI, opsonization index, which is defined as the serum dilution that kills 50% of bacteria.  
^bBRC, baby rabbit complement.  
^cHC, human complement.  
^dHS, human serum.  
^eHS37, HS38, HS39, and HS40, human serum from 4 different healthy unvaccinated subjects.  
^fSerum from rats 278 and 378 prior to vaccination with ExPEC4V vaccine.  
^gSerum from rats 278 and 378 after vaccination with ExPEC4V vaccine.  
^hThe nonspecific killing obtained with 2%, 5%, or 10% BRC or 2% HC was 9%, 23%, 57%, or 25%, respectively. The nonspecific killing obtained with 16.5% BRC was 98%, so OI titers could not be determined.
To further investigate the role of phagocytes in mediating bacterial killing, OPA was performed in the presence of the actin polymerization inhibitor cytochalasin B. The results show that 10 μM cytochalasin B in the presence of HL60 cells could significantly inhibit the killing of *E. coli* serotypes O1A, O2, and O25B (Fig. 2). The overall decrease in percent killing was estimated to be 71.6% across serotypes (*P* = 0.0055; 95% confidence interval [CI], 40%). Notably, the process of phagocytic killing of *E. coli* serotype O6A was completely abrogated by 10 μM cytochalasin B (Fig. 2).

**OPA qualification.** The OPA qualification was performed using an HL60 cell-to-bacterial cell ratio of at least 600:1 and a final human complement concentration of 7%. The performance of the assay was assessed for precision, linearity, trueness, accuracy, assay range, and specificity with pooled serum samples from subjects that had been vaccinated with ExPEC4V (21, 22).

The ranges for linearity (90% CI of the slope, 0.80 to 1.25), precision (coefficient of variation [CV] for repeatability and intermediate precision, ≤50%), accuracy (total error, −65% to 183%), and trueness (relative bias, −30% to 30%) were defined as the target acceptance criteria prior to the qualification and were based on previous experience with cell-based assays. The assay working range was defined as the lowest and highest concentration at which the assay jointly fulfilled the target acceptance criteria for linearity, precision, trueness, and accuracy. The qualification criteria were evaluated with different concentrations using data that passed the system suitability test of maximum killing of ≥85%. Of five serum concentrations assessed, the lowest concentration for serotypes O25B and O2 did not meet the system suitability criteria. Therefore, five concentrations for serotypes O1A and O6A and four concentrations for serotypes O25B and O2 were evaluated. Repeatability (intra-assay) variances and between-run (interassay) variances were estimated by *E. coli* serotype and by concen-

| Complement | Complement concn (%) | Strain (serotype) | NSK[^a] (%) | OI[^b] | Maximum killing (%) |
|------------|----------------------|------------------|-------------|--------|---------------------|
| Pooled human, nonadsorbed | 4 | OC24452 (O1A) | 91 | —[^c] | — |
| | 4 | OC24453 (O2) | 90 | — | — |
| | 4 | OC24454 (O6A) | 85 | — | — |
| | 4 | OC24176 (O25B) | 90 | — | — |
| | 7 | OC24452 (O1A) | 98 | — | — |
| | 7 | OC24453 (O2) | 96 | — | — |
| | 7 | OC24454 (O6A) | 95 | — | — |
| | 7 | OC24176 (O25B) | 99 | — | — |
| Pooled human, preadsorbed, low[^d] | 4 | OC24452 (O1A) | 9 | 189 | 99 |
| | 4 | OC24453 (O2) | 10 | 205 | 94 |
| | 4 | OC24454 (O6A) | 28 | 602 | 96 |
| | 4 | OC24176 (O25B) | 18 | 460 | 100 |
| | 7 | OC24452 (O1A) | 3 | 549 | 100 |
| | 7 | OC24453 (O2) | 8 | 462 | 99 |
| | 7 | OC24454 (O6A) | 76 | 3,053 | 95 |
| | 7 | OC24176 (O25B) | 35 | 605 | 100 |
| Pooled human, preadsorbed, high[^e] | 7 | OC24452 (O1A) | 12 | 193 | 99 |
| | 7 | OC24453 (O2) | 16 | 285 | 99 |
| | 7 | OC24454 (O6A) | 42 | 764 | 100 |
| | 7 | OC24176 (O25B) | 14 | 647 | 100 |

[^a]: NSK, nonspecific killing.
[^b]: OI, opsonization index.
[^c]: —, not determined because NSK was ≥85%.
[^d]: Complement preadsorbed with glutaraldehyde-treated *E. coli* at a ratio of 2 × 10⁹ CFU of each strain (OC24452, OC24453, OC24454, OC24176) per 1 ml complement.
[^e]: Complement preadsorbed with glutaraldehyde-treated *E. coli* at a ratio of 2 × 10⁹ CFU of strain OC24452 or OC24453, 7 × 10⁹ CFU of strain OC24454, and 4 × 10⁹ CFU of strain OC24176 per 1 ml complement.
tration using a linear mixed model fitted on the log_{10} titers, including assay run (a combination of runs, days, and operators) as a single random factor (series). To obtain the variance components by *E. coli* serotype across concentrations, a second model was fitted by including series as the random factor and concentration as the fixed factor. Repeatability variances and between-run variances were determined for each model. Intermediate precision variance is the sum of the repeatability variance and between-run variance. Repeatability standard deviations and coefficients of variation (CVs) and intermediate precision standard deviations (SDs) and CVs were estimated. A CV for the repeatability and intermediate precision of the assay per concentration and overall of $\leq 50\%$ was considered acceptable.

The results show that for serotype O1A the main source of variability was from assay run to assay run (CV, 9% to 31%), whereas for serotypes O2 and O6A, the main source of variability was repeatability (CV, 8% to 18%) (Table 3). For serotype O25B, both sources of variability (repeatability and run to run) contributed equally to the total variation (Table 3).

For each serotype and concentration, the CVs for intermediate precision reached a maximum of 33% for serotype O1A, 31% for serotype O2, 17% for serotype O6A, and 22% for serotype O25B (Table 3). Estimates of repeatability and intermediate precision were computed for each serotype across the whole range of concentrations tested (Table 3). The CVs for intermediate precision were $\leq 23\%$ for all serotypes.

Linearity was assessed by plotting the log_{10}-transformed titers against the log_{10} theoretical titer expected from serial dilution of the titer observed with the first concentration tested for each serotype. A linear regression model was fitted to the

**FIG 1** Evaluation of the contribution of key components of OPA. OPA was performed in the presence of all components (standard condition), including HL60 cells (phagocytes), *E. coli* bacterial cells, complement, and human serum antibodies (filled circles) or with the removal of phagocytes (open circles), complement (filled diamonds), serum antibodies and complement (open diamond), or serum antibodies and phagocytes (half-filled circles). The results are expressed as the mean number of CFU per milliliter ± SEM determined for duplicates of a 3-fold dilution curve. The assay was performed with human complement (7%) from a single donor. The area under the curve (AUC) for each condition and serotype was calculated, and pairwise comparisons were performed between conditions, omitting key assay components and the reference condition (serum, bacteria, complement, HL60 cells) while correcting for serotype. Dunnett’s method was applied to correct for multiple comparisons. *P* was $<0.0001$ for all four conditions tested.
log$_{10}$ titers as a function of the log$_{10}$ theoretical titers for each serotype. Linearity was considered acceptable if the 90% confidence interval (CI) of the slope of each strain was within the range of 0.80 to 1.25.

Regression modeling indicated the linearity of the assay (Fig. 3), with the 90% CI of the slopes of all serotypes being fully included in the predefined acceptance limits of 0.80 to 1.25 (Table 4).

Trueness was assessed by means of relative bias since the true concentration of the samples was unknown. The relative bias was calculated as the difference between the geometric mean of the observed titers and the theoretical titer divided by the geometric mean of the theoretical titer on the original scale, expressed as a percentage. The geometric means of the observed titers were obtained from the linear mixed model by concentration and serotype after back calculation of the mean log$_{10}$ titers to the original scale. Trueness was considered to be within acceptable levels if the relative bias at each concentration was within the range of $-30\%$ to $30\%$. The accuracy or total error of the results generated by the OPA was estimated by calculating the 90% beta-expectation tolerance interval on the log$_{10}$ titers by including series as a random effect and using the linear mixed model described above for each concentration. The 90% beta-expectation tolerance interval expressed as relative error at each theoretical titer was acceptable if the values fell within the range of $-65\%$ to $183\%$.

Data analysis showed that the relative bias was always less than approximately 30% for all serotypes and concentrations evaluated (data not shown). The 90% beta-expectation tolerance intervals fell completely within the acceptance criterion range of $-65\%$ to $183\%$ for all serotypes and concentrations evaluated (Fig. 4).

The assay range for each serotype was determined to be the lowest and highest concentrations evaluated during the qualification in which the assay showed acceptable linearity, precision, trueness, and total error. The lower and upper limits of the assay range were set at the theoretical titers. For serotypes O1A and O6A, five

![FIG 2 Phagocytosis is required for E. coli killing in the OPA. HL60 cells were incubated for 30 min with cytochalasin B (CytB; 10 μM) or dimethyl sulfoxide (DMSO; vehicle). The opsonization index (OI) obtained for the standard condition (−, black bars) was set as 100% killing, and the tested conditions were calculated as follows: percent killing = (OI for the test condition/OI for the standard condition) × 100. A paired t test was used to compare the percent killing between cytochalasin B treatment and the reference condition (no treatment) across serotypes (P = 0.0055; lower and upper 95% confidence limits [CL], −100% and −40%, respectively).](image-url)
concentrations fell within the assay working range, and for serotypes O2 and O25B, four concentrations fell within the assay working range (Table 5).

The specificity of ExPEC4V-induced serum antibodies for each of the four E. coli serotypes was demonstrated by preadsorbing serum with E. coli cells of each serotype: specificity was shown by the selective decrease in OI titers to the homologous serotype compared to the titers to the remaining three heterologous serotypes.

Nonspecific activity and specific activity were defined as the percent reduction in the OI value of heterologous and homologous serum preadsorptions, respectively. Vaccine-induced antibodies were determined to be specific to each of the serotypes if the reduction of the OI value was \( \geq 70\% \) for homologous serum preadsorption OPA combinations.

Preadsorption of serum pools with homologous O-serotype E. coli cells yielded OPA titer reductions of \( \geq 99.6\% \), while preadsorption of serum pools with heterologous O-serotype E. coli cells yielded OPA titer reductions of \( \leq 20.6\% \) (Table 6).

**Evaluation of ExPEC4V immunogenicity using the qualified OPA.** The qualified OPA was used to assess the immunogenicity of a 4-valent E. coli bioconjugate vaccine (ExPEC4V) administered to humans in a phase 1 study of women with recurrent UTIs (10). Representative OI data for the sera from 10 subjects vaccinated with ExPEC4V and 2 subjects vaccinated with a placebo are shown in Table 7. For all four O serotypes, vaccine-mediated increases in titers (1.2- to 241.6-fold) were seen when the titers from day 1 (prevaccination) were compared to those from day 30 for the vaccinated subjects, while the titers for the placebo-vaccinated subjects showed little or no change when those on day 1 were compared to those on day 30.

**DISCUSSION**

The World Health Organization recognizes the demonstration of the OPA titer to be a key secondary readout for the clinical efficacy of PCVs and requires measurement of...
the OPA titer in a subset of samples to support licensure of new vaccines (23). We drew on parallels between the production and mechanisms of immune protection between ExPEC vaccines and PCVs to develop an OPA suitable for the evaluation of human serum in clinical trials of a bioconjugate vaccine (ExPEC4V). To date, guidelines for the development of ExPEC vaccines and requirements for the demonstration of immunogenicity are not available. However, OPA is expected to be an important readout to demonstrate immunogenicity to support the licensure of ExPEC vaccines.

Measurement of antibodies using ELISA is a well-established method of evaluating the immunogenicity of vaccines. However, the OPA more accurately reflects the in vivo protective capacity of these antibodies; the OPA result correlates more closely with clinical protection than the ELISA result in infants vaccinated with a PCV (12–14) and is likely to be a surrogate marker of protection for other bacteria, such as E. coli, for which opsonophagocytosis is an important clearance mechanism in the human host (24–26).

The information in the literature regarding E. coli OPAs is minimal in comparison to that regarding other bacteria, such as pneumococci, and typically comes from studies involving the use of neutrophils as effector cells, as described by Cross et al. (17). The use of HL60 cells in an E. coli killing assay is rarely reported (27), and there are no reports, to our knowledge, of a qualified E. coli OPA using HL60 cells. In order to avoid

![FIG 3 Assay linearity evaluation. Plots of the log_{10} titers versus log_{10} theoretical titers per serotype are shown. Dashed line, the unit line between the log_{10} titers and the log_{10} theoretical titers.](image)

| Serotype | Slope | 90% CI^b |
|----------|-------|----------|
|          | Lower | Upper    |
| O1A      | 1.02  | 1.01     | 1.04
| O2       | 0.97  | 0.94     | 1.00
| O6A      | 1.00  | 0.99     | 1.01
| O25B     | 1.01  | 0.98     | 1.04

^aLinearity experiments used human serum.
^bCI, confidence interval.
the technical challenges and variability associated with the isolation and use of human neutrophils and to better align with other OPAs already recognized by regulatory authorities (28), the previously described pneumococcal OPAs (14, 19) that use HL60 cells were adapted for use with *E. coli*. The original assay using BRC proved insensitive to differences between pre- and postvaccination serum samples. Human complement improved the ability of the assay to differentiate between pre- and postvaccination samples but required the addition of a preadsorption step to reduce the high levels of NSK activity against *E. coli* strains, likely due to the presence of preexisting antibodies (17). The ratios of HL60 cells to bacteria were optimized, and growth conditions for bacterial enumeration were modified to suit the characteristics of *E. coli*.

Qualification steps showed that for serotypes O1A, O2, O25B, and O6A, the accep-

![Graphs showing total error profiles for different concentrations per serotype](image)

**TABLE 5** Assay working range

| Serotype | Log₁₀ scale | Original scale |
|----------|-------------|----------------|
|          | LLOQ⁻ | ULOQ⁻ | LLOQ  | ULOQ  |
| O1A      | 1.176 | 3.584 | 15    | 3,841 |
| O2       | 1.620 | 3.717 | 42    | 5,208 |
| O6A      | 1.255 | 3.164 | 18    | 1,458 |
| O25B     | 1.070 | 2.502 | 12    | 317   |

⁻Assay working range experiments used human serum.

⁻⁻LLOQ, lower limit of quantification.

⁻⁻⁻ULOQ, upper limit of quantification.
tance criteria for precision, trueness, linearity, and total error were fulfilled within the working range of the assay. The assay was shown to be specific for all serotypes: OPA titers were reduced by ≥ 99.6% when serum pools were preincubated with *E. coli* cells of the homologous serotype, while OPA titers were reduced by ≤ 20.6% when serum pools were preadsorbed with *E. coli* cells of heterologous serotypes. These results are consistent with the predetermined targets and therefore qualified the OPA for its purpose and support the serotype specificity of the antibodies generated by ExPEC4V.

Further work to be done includes investigation of a potential protective OPA titer threshold for ExPEC infections. It is unlikely that the same threshold will apply to all serotypes or to different clinical syndromes, such as UTIs in healthy women versus bacteremia in elderly individuals. Future studies to evaluate the levels of anti-*E. coli* functional antibodies present in the serum of subjects with a history of recurrent urinary tract infection or bacteremia in comparison with those in the serum of healthy controls could support the establishment of a correlate of protection for ExPEC infections.

Standardization of OPAs between laboratories has been a significant challenge in the use of OPAs for PCVs (14, 29) but was fundamental for PCV clinical development. Therefore, interlaboratory standardization of the *E. coli* OPA will become important not only for vaccine studies but also for natural immunity studies and will support the further development of an ExPEC vaccine. Progress on this front and existing collaborations between manufacturers, regulatory agencies, and WHO can be applied to new *E. coli* OPAs.

The qualified *E. coli* OPA was used to evaluate human serum from a phase 1 clinical trial and successfully demonstrated a vaccine-mediated increase in OI titers at 30 days postvaccination (10); this increase in titers was similar to that reported for other conjugate vaccines, such as the pneumococcal 7-valent conjugate vaccine Prevnar7 (30, 31). In conclusion, we have developed an OPA based on the pneumococcal OPA

**TABLE 6** OPA specificity expressed as a percentage of OI² reduction

| Serotype | Specificity (OIA reduction in OI) | OI | O2 | O6 | O25 |
|----------|--------------------------------|----|----|----|-----|
| O1A      | 99.6                          | 17.2| 12.3| 20.6|
| O2       | 6.5                           | 98.9| 14.2| 3.3 |
| O6A      | 0.5                           | 3.3 | 100 | -11.3|
| O25B     | 8.8                           | -0.2| -32.0| 100 |

²OI, opsonization index. Specificity experiments used human serum.

**TABLE 7** OI values determined using the qualified OPA with human serum from a phase 1 study evaluating ExPEC4V

| Subject no. | Vaccine | OI² | OIA Positive OIA Positive OIA Positive OIA Positive OIA Positive |
|-------------|---------|-----|---------------------|---------------------|---------------------|---------------------|---------------------|
|             |         | Serotype O1A | Serotype O2 | Serotype O6A | Serotype O25B |
|             |         | Day 1 | Day 30 | Day 1 | Day 30 | Day 1 | Day 30 | Day 1 | Day 30 |
| 1           | ExPEC4V | 249 | 1,451 | 340 | 12,922 | 491 | 7,626 | 77 | 226 |
| 2           | ExPEC4V | 149 | 1,151 | 228 | 8,897 | 497 | 1,292 | 338 | 606 |
| 3           | ExPEC4V | 42  | 3,578 | 167 | 23,210 | 406 | 3,784 | 210 | 3,423 |
| 4           | ExPEC4V | 122 | 9,641 | 560 | 9,824 | 541 | 4,559 | 6 | 1,450 |
| 5           | ExPEC4V | 126 | 15,207 | 674 | 26,244 | 1,292 | 3,390 | 476 | 1,265 |
| 6           | ExPEC4V | 177 | 1,121 | 484 | 15,609 | 812 | 2,140 | 86 | 184 |
| 7           | ExPEC4V | 147 | 5,889 | 128 | 10,371 | 509 | 2,492 | 23 | 87 |
| 8           | ExPEC4V | 361 | 510 | 5,385 | 10,318 | 508 | 13,407 | 212 | 1,133 |
| 9           | ExPEC4V | 15  | 477 | 21 | 6,135 | 2,091 | 9,665 | 4,609 | 5,360 |
| 10          | ExPEC4V | 173 | 379 | 187 | 7,284 | 999 | 2,759 | 6 | 81 |
| 11          | Placebo | 229 | 197 | 374 | 304 | 353 | 392 | 265 | 208 |
| 12          | Placebo | 197 | 197 | 230 | 210 | 404 | 457 | 320 | 249 |

²OI, opsonization index. OI titers at or below the lower limit of quantification were replaced with the respective values of one-half the lower limit of quantification. Day 1 is prevaccination.
suitable for multiple *E. coli* serotypes and have demonstrated the ability of this assay to measure a functional antibody response in human serum from a phase 1 clinical trial of ExPEC4V (10).

**MATERIALS AND METHODS**

**Bacterial strains.** *E. coli* strains OC24452 (O serotype O1A), OC24453 (O serotype O2), OC24454 (O serotype O6A), and OC24176 (O serotype O25B) were used in the OPA. These strains are clinical isolates that were selected on the basis of their subtype specificity and their sensitivity in discriminating pre- from postvaccination serum responses.

The O serotype of each isolate was confirmed by agglutination using a single *E. coli* O-serotype-specific antisera (Statens Serum Institut, Denmark) and PCR using O-serotype-specific primers described by Fratamico et al. (32) and Li et al. (33). Assay working stocks were created on the basis of the method described by Burton and Nahm (34). In brief, bacteria from master stocks were streaked onto blood agar plates and incubated overnight at 37°C in ambient air. Sterile Todd-Hewitt broth (Becton Dickinson, Sparks, MD) with 0.5% yeast extract was inoculated with multiple colonies, which were incubated at 37°C in ambient air with shaking at 110 rpm until the optical density at 600 nm was 0.6 to 0.9. Working assay stocks were stored until needed by cryopreservation with 15% glycerol at −80°C.

In the OPA, the enumeration of bacterial colonies was performed by spotting bacteria on glycerol yeast extract agar (GYA) containing 2,3,5,-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich). The GYA medium comprised M9 minimal salts (Becton Dickinson), MgSO₄ (Sigma-Aldrich), yeast extract (Becton Dickinson), glycerol (Sigma-Aldrich), and Bacto agar (Becton Dickinson).

**Phagocytes.** HL60 cells were obtained, propagated, and differentiated as described previously (19), except that the differentiation with dimethyl formamide was done for 3 days. Cells were assessed for their suitability for use in the OPA by flow cytometry, as described previously (19). These cells were used as the source of phagocytes for the OPA.

**Serum samples.** Human serum (HS) from healthy subjects was obtained from Sigma-Aldrich (St. Louis, MO; sample HS) or Biological Specialty Corporation (Colmar, PA; samples HS37, HS38, HS39, HS40, and HS58). Rat serum samples were obtained from preclinical studies in which animals were vaccinated with ExPEC4V (35). Human serum was also obtained before and after vaccination (12 paired samples, n = 24) from subjects participating in a phase 1 clinical trial assessing the safety and immunogenicity of a bioconjugate *E. coli* vaccine (ClinicalTrials.gov registration number NCT02289794) (10). For OPA qualification, human serum samples obtained from 4 subjects after vaccination with ExPEC4V were pooled. Rats were maintained, immunized, and bled at the animal facility of Eurogentec SA, and studies were conducted according to institutional guidelines. The phase 1 study and all protocol amendments were reviewed and approved by the independent ethics committees of each site and by the Swiss Agency for Therapeutic Products (Swissmedic).

**Complement source.** The complement initially used for OPA development was from 3- to 4-week-old rabbits (Pel-Freez, Rogers, AR, or Cedarlane, Burlington, NC). OPA qualification used pooled human complement (Cedarlane) from three different donors. To reduce nonspecific killing (NSK), human complement was preadsorbed with glutaraldehyde-treated *E. coli* bacteria at a ratio of 1 ml complement per 2 × 10⁹ CFU of OC24452 (O1A), 2 × 10⁹ CFU of OC24453 (O2), 7 × 10⁶ CFU of OC24454 (O6A), and 4 × 10⁹ CFU of OC24176 (O25B). Glutaraldehyde-treated *E. coli* bacteria and human complement were incubated for 60 min at 4°C with gentle rocking. After incubation, the glutaraldehyde-treated *E. coli* bacteria were removed by centrifugation (16,748 g, 5 min), 4°C and the complement was either used immediately or aliquoted and stored at −80°C.

**OPA procedure.** The assay was based on the OPA described by Romero-Steiner et al. (14) and Burton and Nahm (19) for the evaluation of pneumococcal antibodies. Assays were carried out using round-bottom 96-well plates (Costar). Serum samples were heat inactivated by incubation at 56°C for 30 min and added to the assay plate (20 μl/well) at a starting dilution of 1:12, followed by 7 additional serial dilutions in 3-fold steps. All serum samples were tested in duplicate. Frozen stocks of *E. coli* were thawed, washed with opsonization buffer B (OBB; Hanks’ balanced salt solution [HBSS]) with magnesium, calcium, and 0.1% gelatin) by centrifugation (12,000 rpm, 2 min), diluted to −10⁷ CFU/ml, and dispensed at 10 μl/well. After 30 min of incubation at room temperature, 5.6 μl of complement, 36.8 μl of HL60 cells, and 7.6 μl of OBB were added to each well. Before use, HL60 cells were washed twice with HBSS by centrifugation (350 × g, 5 min) and adjusted in OBB to 1.6 × 10⁷ cells/ml. The plates were incubated for 60 min at 37°C in 5% CO₂, with shaking (Bellco Biotechnology, Vineland, NJ) at 700 rpm. Afterwards, the plates were placed on ice for 20 min and 10 μl of the final reaction mixture was spotted onto GYA plates containing TTC. The GYA plates were incubated for 15 to 16 h at 33°C.

The number of bacterial colonies on the agar plates was enumerated by digitally photographing the plates (Nikon D3200) with colony-counting software (NICE, U.S. National Institute of Standards and Technology [http://www.vaccine.uab.edu/uploads/mdocs/NICE.pdf]). Opsonization index (OI) values (referred to here as titers) were defined as the serum dilution that killed 50% of the bacteria and were determined using the Opsotitter3 Excel-based program developed at the University of Alabama at Birmingham, Birmingham, AL, USA.

**OPA qualification.** The performance of the assay was assessed for repeatability, intermediate precision, linearity, total error, assay range, and specificity with the pooled human serum samples from subjects vaccinated with ExPEC4V (21, 22).

The assay target acceptance criteria were defined as follows: linearity, 90% CI of the slope of 0.80 to 1.25; precision, CV for repeatability and intermediate precision of ≤50%; accuracy, total error of −65%...
was calculated. In addition, a model was fitted to the log10 AUC by including serotype and treatment as fixed effects. Pairwise comparisons between treatment conditions were performed by omitting key components of the assay and the reference condition (serum, bacteria, complement, and HL60 cells). Dunnett’s method was applied to correct for multiple comparisons. A paired t test was used to evaluate the effect of cytolysin B treatment on the percent E. coli killing compared to the amount of E. coli under the reference condition (no treatment) across serotypes. P values of <0.05 were considered significant.

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